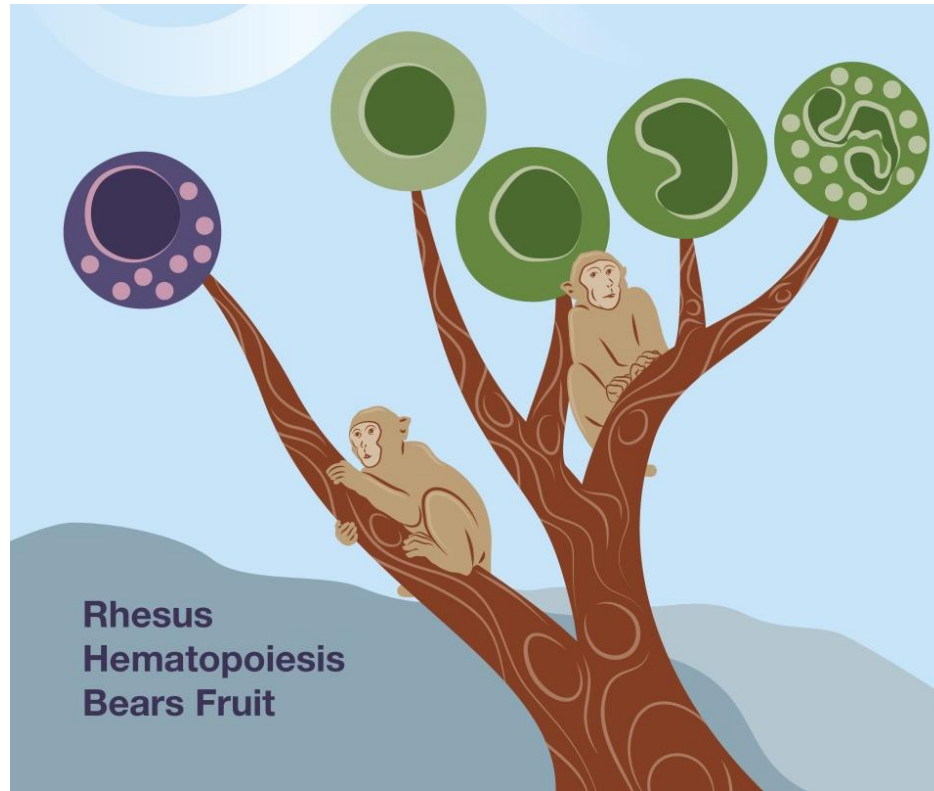


Hematopoiesis from the Bench to the Bedside



Cindy Dunbar, MD

Translational Stem Cell Biology Branch

Disclosures

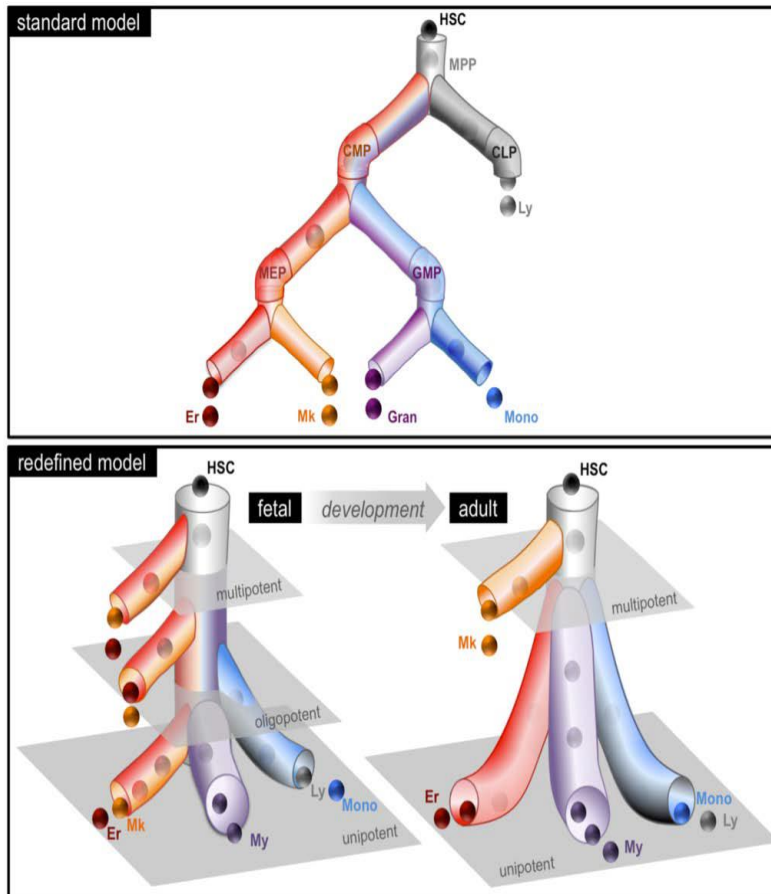
- Drug and clinical trial funding from Glaxo/Smith/Kline and Novartis
- These clinical trials initially involved off-label use of eltrombopag, but these indications have since received FDA approval

Learning Objectives

- Understand the clonal dynamics of hematopoietic stem cells with clinical relevance
- Become aware of new pharmacologic and genetic therapies targeting hematopoietic stem cells

Interrogating Hematopoiesis at a Clonal Level

Mapping Hierarchies and Output of Individual HSPC



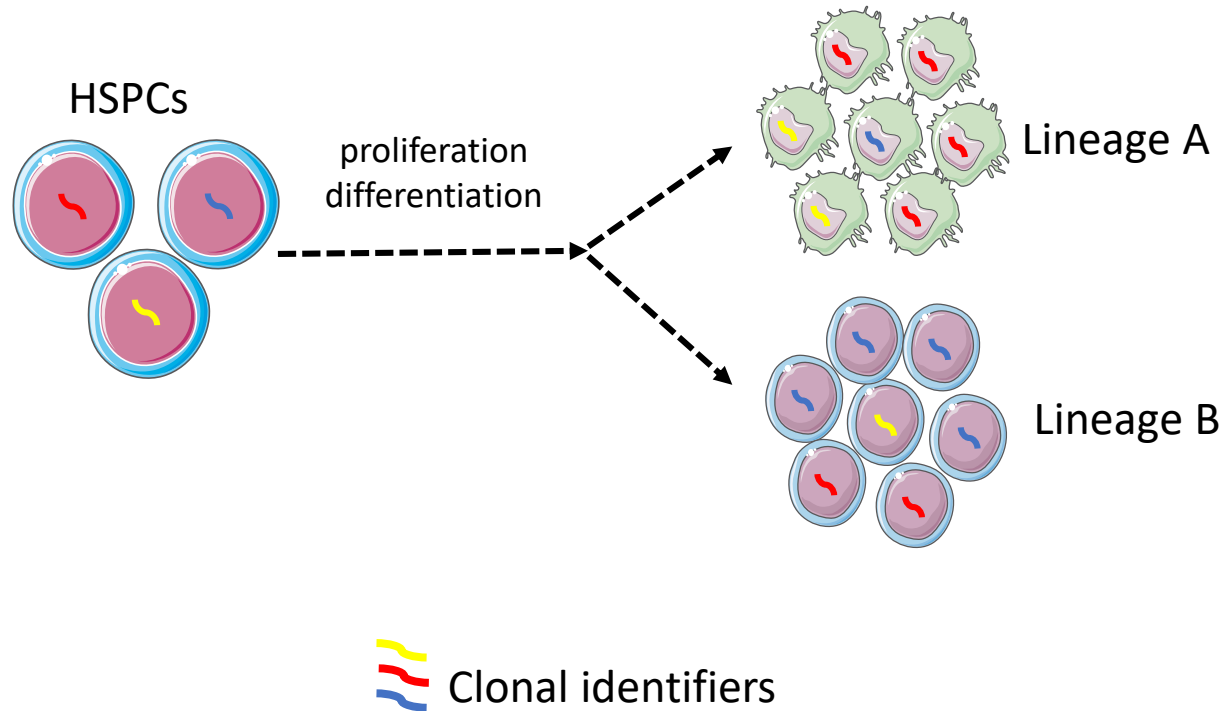
- Limit dilution transplantation into irradiated mice
- What an HSPC cell CAN do under profound replicative stress

versus

- What HSPC's DO do under more physiologic or clinically-relevant conditions

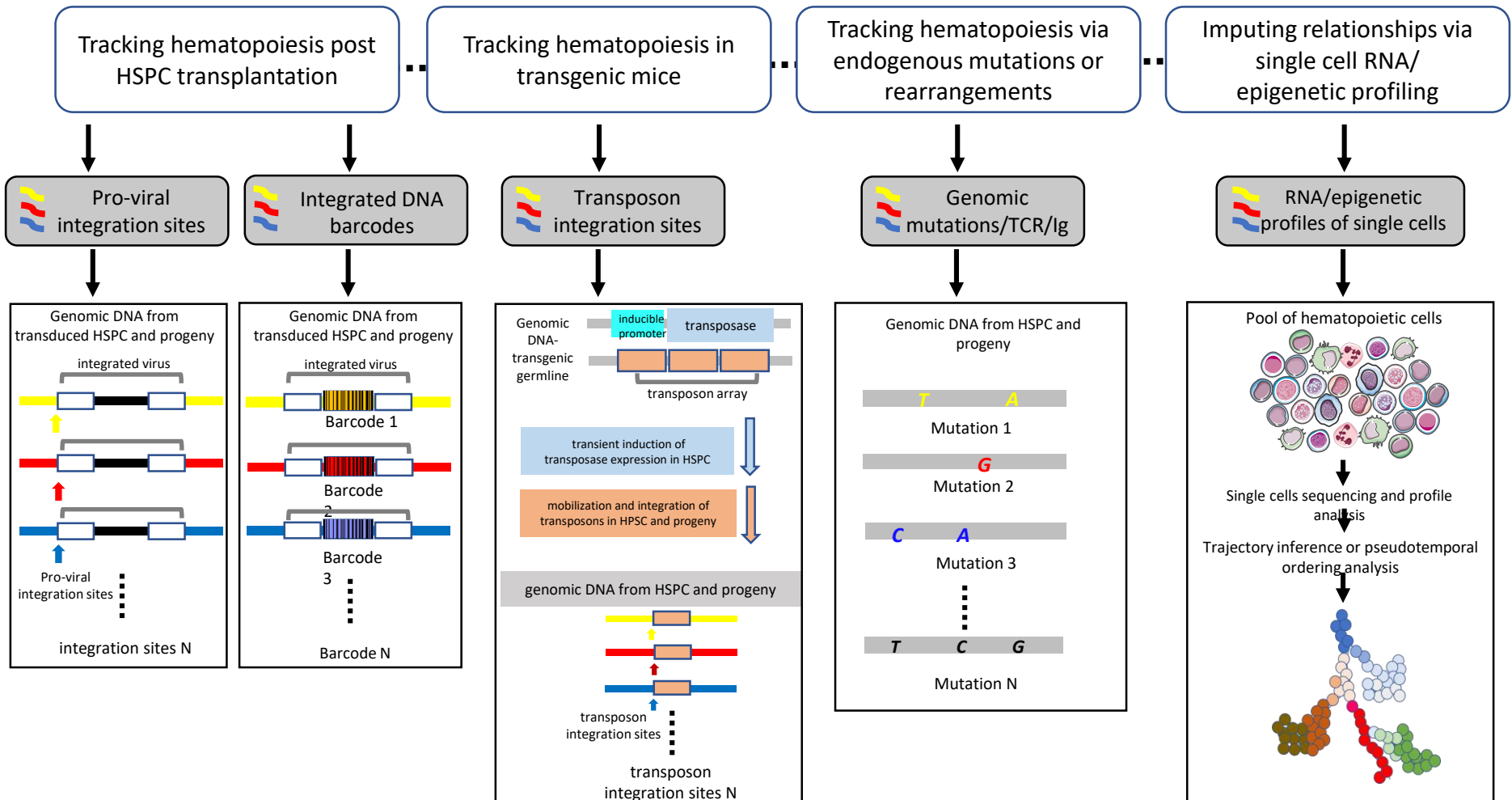
Interrogating Hematopoiesis at a Clonal Level

Mapping Hierarchies and Output of Individual HSPC



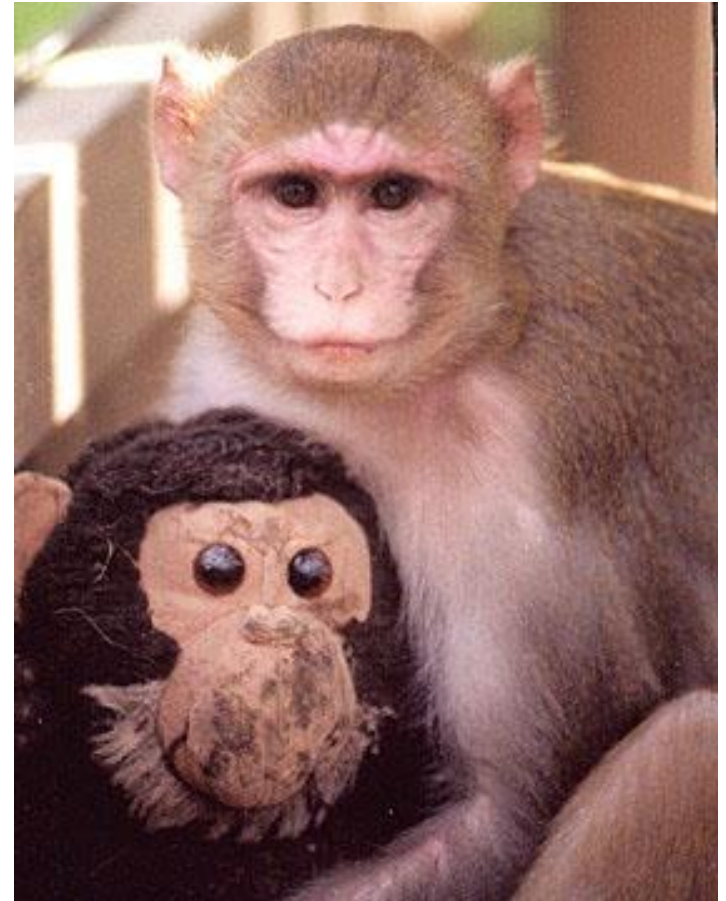
Interrogating Hematopoiesis at a Clonal Level

Mapping Hierarchies and Output of Individual HSPC

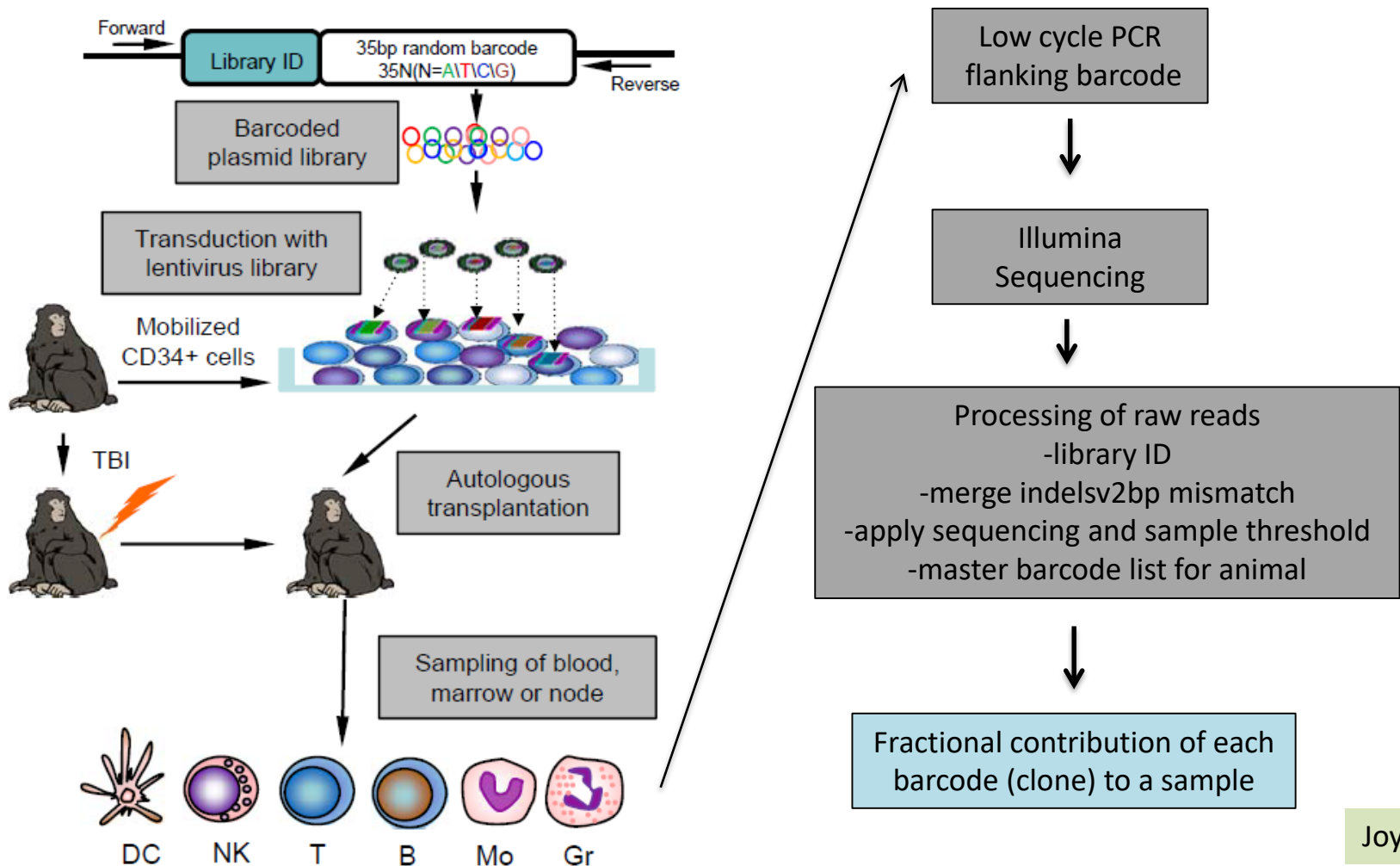


Rhesus Macaques as Models for Human Hematopoiesis

- Lack of predictive value of murine models
- Life span (up to 30-35 years) and size (5-20 kg) relevant for humans
- HSPC and immune cells homologous to human
 - Phenotype
 - Frequency
 - Telomere lengths
 - Marrow and immune tissue architecture/function
- 30 years of rhesus HSPC gene transfer, hematopoiesis, transplantation and vaccine studies mirror human outcomes

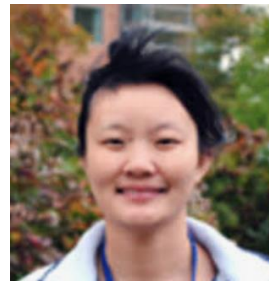


Barcoding of HSPC in Rhesus Autologous Transplant Model

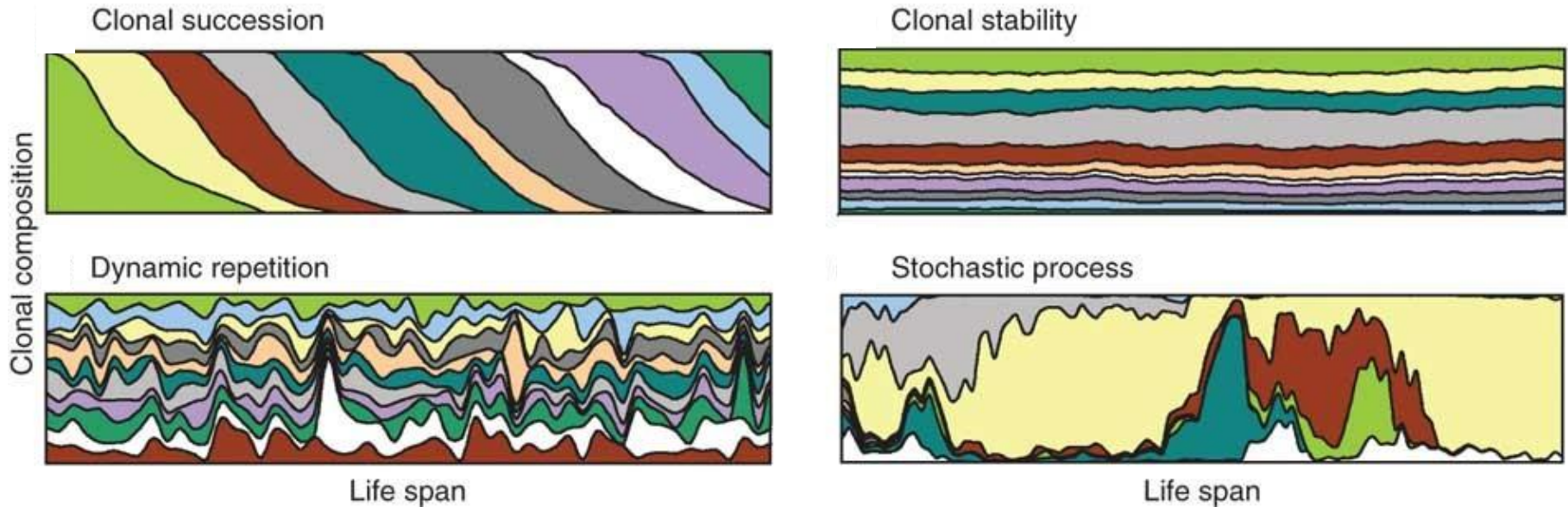


- One barcode=one transduced progenitor or stem cell
 - Library diversity must be adequate for the # of engrafting cells
- Quantitative-barcode read fraction = fractional contribution of clone

Joy Wu



Output from Stem Cells Long-Term: Stability or Succession



From Bystrykh et al, Nat Meth 2012

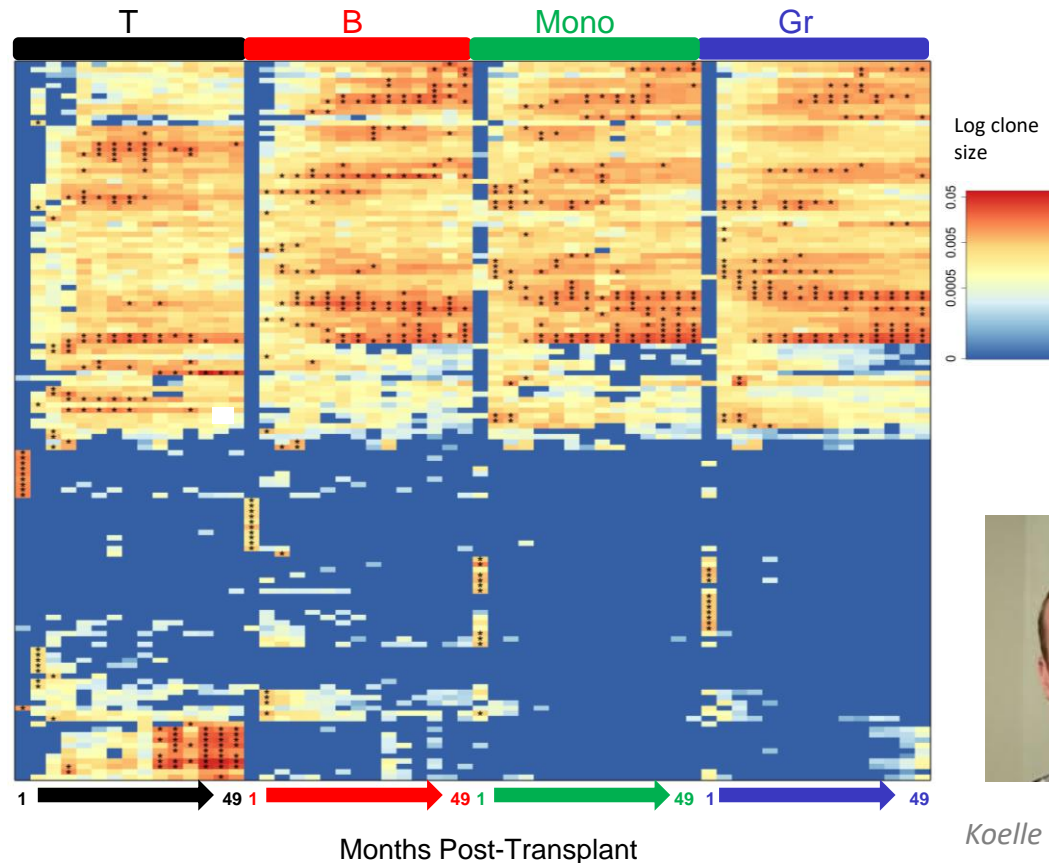
- Clinical relevance
 - Stability of gene therapies targeting HSPCs
 - Impact of aging on HSPC pool and progression towards transformation
 - Impact of HSPC-depleting therapies
- Evidence for clonal succession
 - Early gene transfer studies in mice using primitive clone tracking approaches
 - Transposon mobilization studies in mice (Sun et al Nature, 2014)
 - Molecular clock studies in mice

Long-Term Hematopoiesis Derives from Stable Multipotent Clones

Rows = individual clones
Clustered by correlations

Columns=samples over time

Focus on top contributing clones

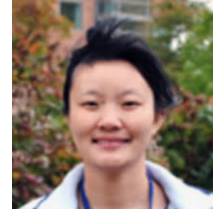


Koelle et al, Blood , 2017

- Short term HPSCs contribute for 1-2 months, lineage restricted
- Contributions from multi-potent long-term HSPCs stable for up to 7 years
- Tens of thousands of HSPCs contributing long-term-calculate frequency and numbers of HSPC per animal
- Supported by human data
 - Stability of natural somatic mutations over time (Lee-Six et al, Nature, 2018)

Additional Insights from Macaque Clonal Tracking

- Sustained geographic restriction of HSPC clones
 - Up to several years
 - Local dispersion of self-renewing HSPCs
 - Marrow exit normally a death pathway?
 - Implications for how we look for mutations clinically



Wu et al JEM, 2018

- Natural killer cell life histories
 - Subsets of mature NK cells clonally-expand and persist independent of ongoing production from HSPCs for many years
 - Wax and wane over time, responding to environmental stimuli such as CMV
 - Clones are KIR-restricted, and may explain functional NK cell memory



Wu et al Cell Stem Cell, 2014

Wu, Espinoza et al Sci Immunol 2018

Truitt et al Front Imm, 2020



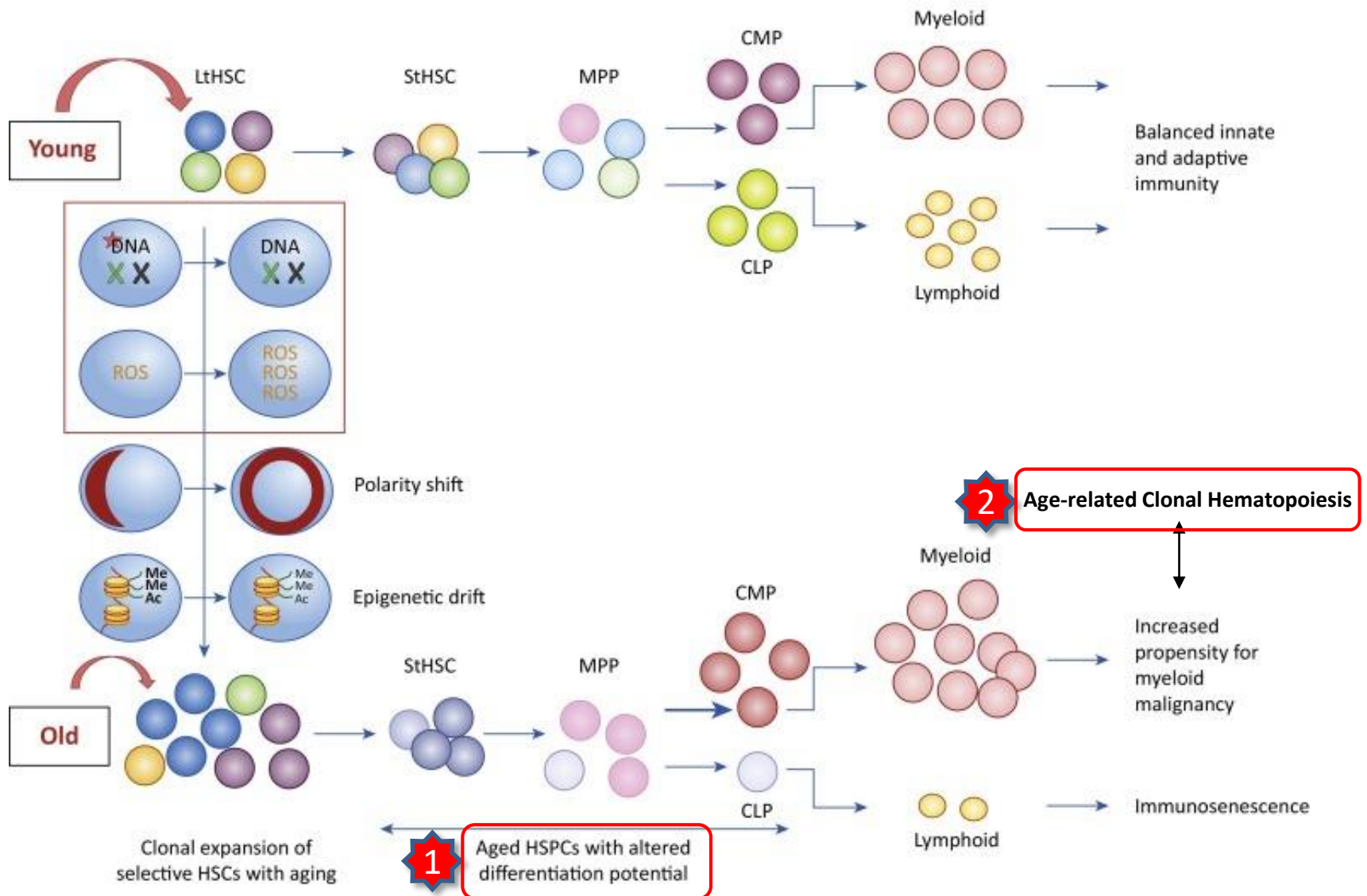
- Genotoxicity of integrating viral vectors used for gene therapies
 - Detection of premalignant clonal expansions
 - Comparison of vector designs
 - Detailed mapping of the process of overt clonal transformation



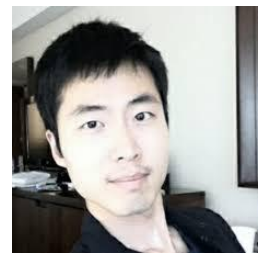
Yabe et al MTCD, 2019

Espinoza et al Mol Ther, 2019

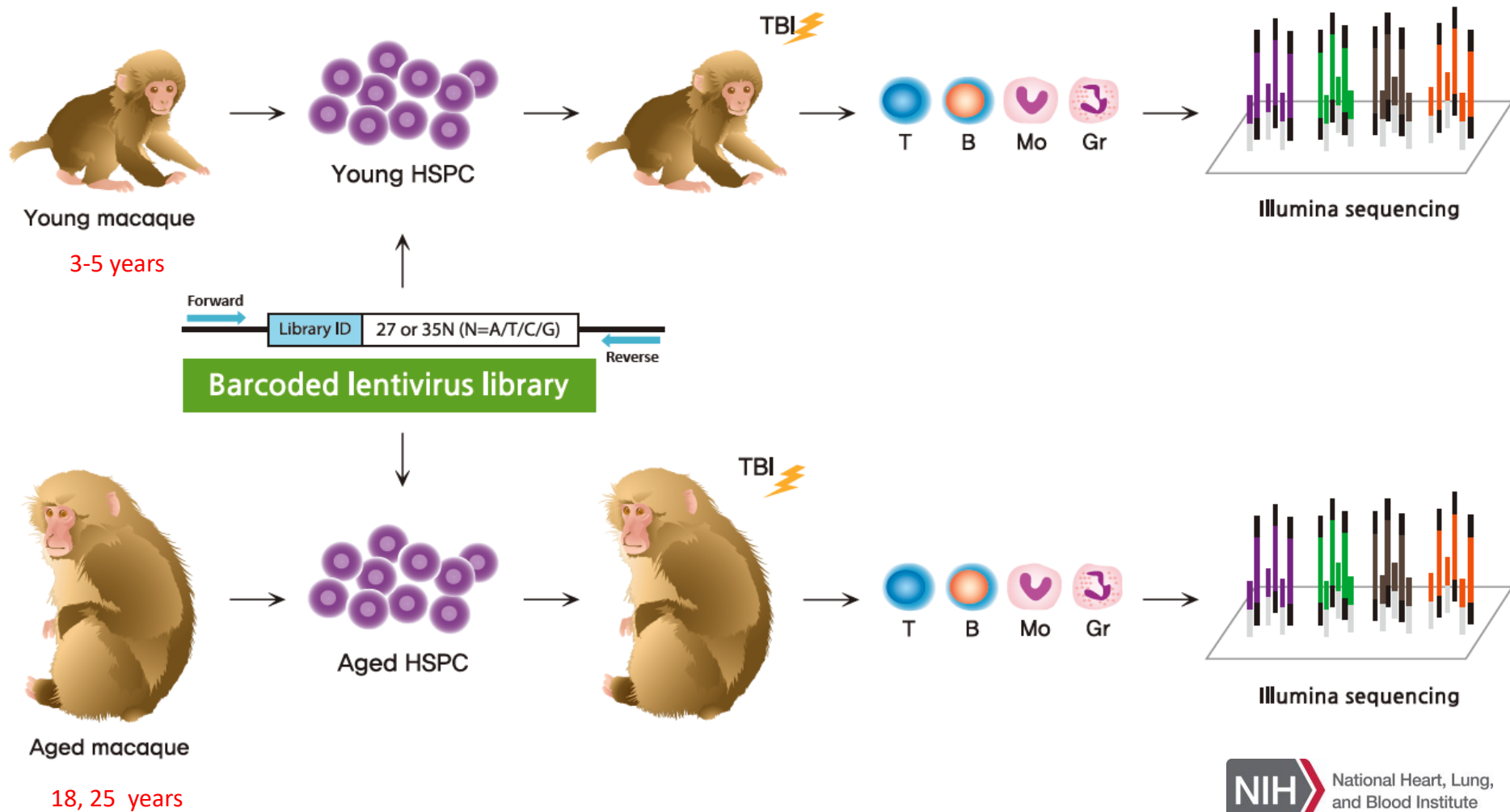
Hematopoietic Stem Cell Aging



Clonal Tracking in Young vs Aged Macaques



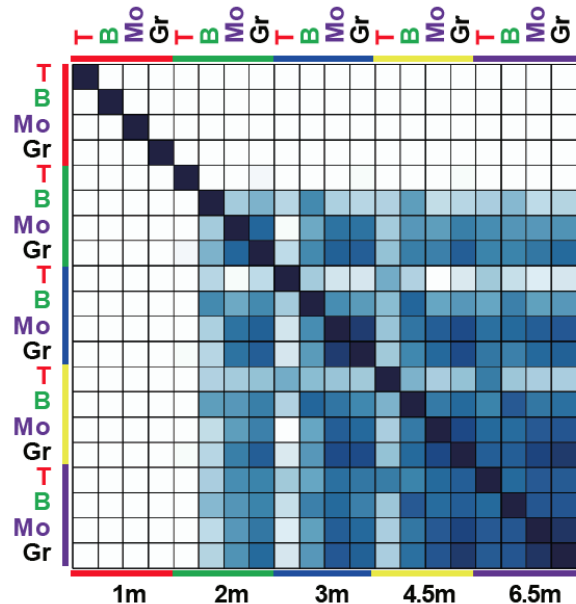
Kyung-Rok Yu



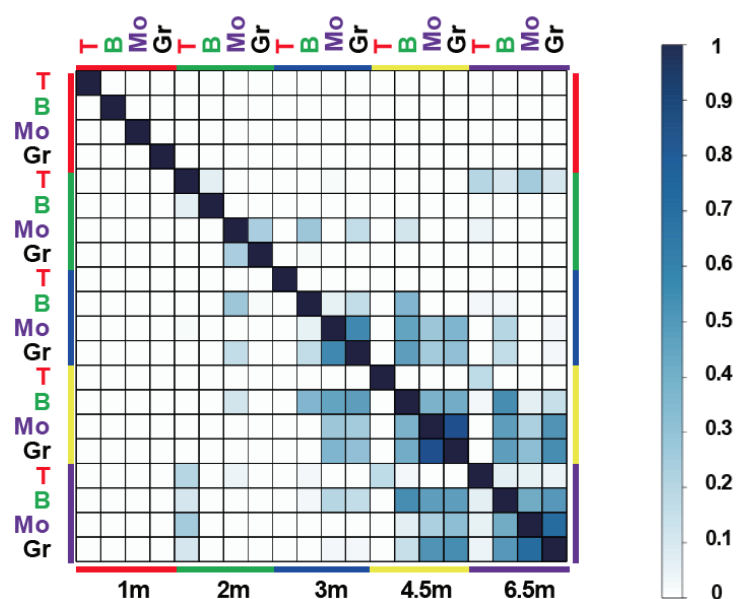
Aged macaques showed delayed emergence of multipotent clones



ZH33 (3 yrs, Young)



RQ859 (25 yrs, Aged)



- Thousands of contributing clones mapped over time and across lineages
- Pearson correlations between clonal contribution levels at different time points and across lineages

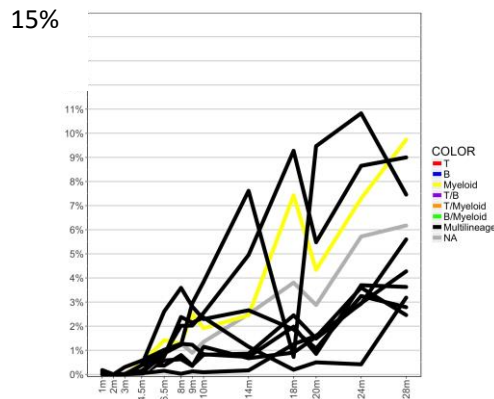
Expansion of Individual Multipotent, Myeloid- and B-biased Clones in Aged Macaques

The 10 most abundant clones at the latest time point were tracked back over time

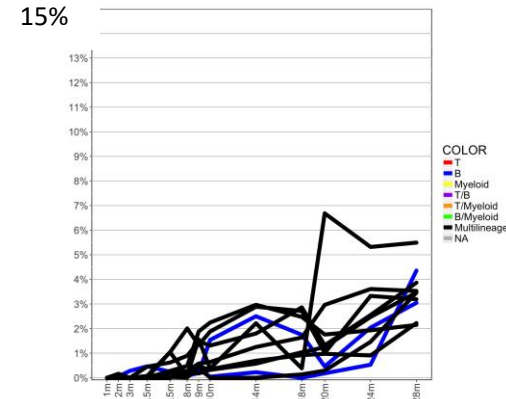


Aged

Granulocytes



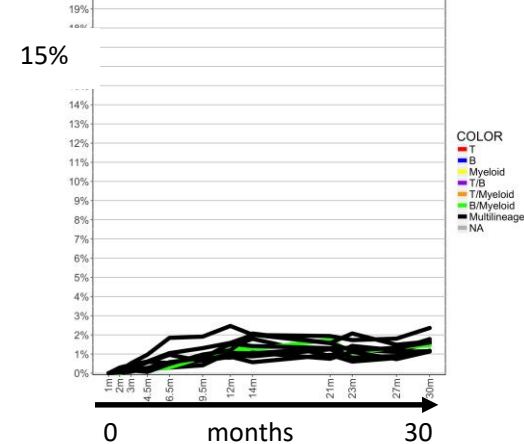
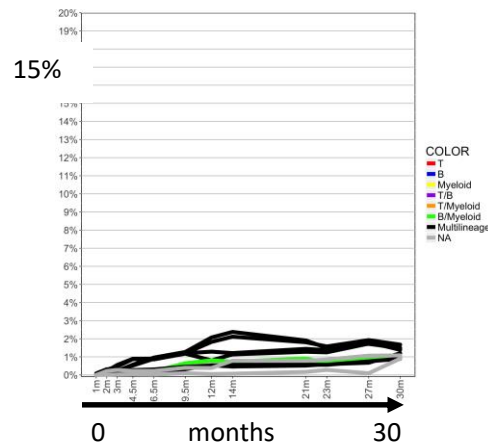
B Cells



- Multipotent
- B-biased
- My-B biased
- Myeloid-biased



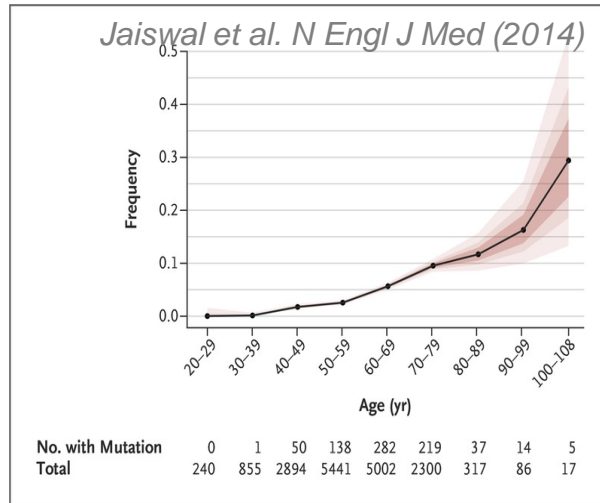
Young



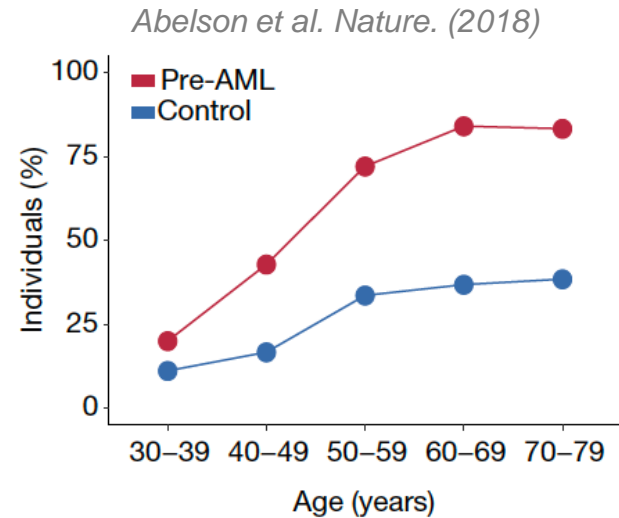
- Model for clonal hematopoiesis of aging (ARCH or CHIP)

Age-Related Clonal Hematopoiesis (ARCH)

Clonal Hematopoiesis of Indeterminate Prognosis (CHIP)



Error-corrected
sequencing



- **Acquired somatic mutations in human blood cells/HSPC**
 - First reported in large population-based sequencing studies in 2014
 - Increasing in frequency and VAF (mutant allele fraction) with aging
- **Defined as “clonal hematopoiesis” by clinicians or for studies if:**
 - No evidence of a hematologic neoplasm
 - Normal blood counts
 - Somatic mutations VAF > 2.5%
- **Mutations in DNMT3A, TET2, ASXL1 genes most common**
 - Heterozygous loss-of-function
 - Epigenetic regulators previously linked to myeloid neoplasia
 - Increased risk of hematologic malignancies
 - Increased risk of cardiovascular disease

Models for Human Clonal Hematopoiesis

Human ARCH/CHIP

- Absence of cytopenias/clinical abnormalities - limited sampling/tissues available
- Long-term follow-up of hematopoietic dynamics and function of mutant clones challenging

Murine models

- No natural ARCH-type mutations detected in aged mice
- Engineered mice can go to MPD or AML quickly
- Limited follow-up period
- Different HSPC properties



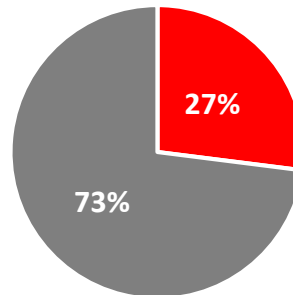
Rhesus macaque model

- High sequence homology
- Similar marrow architecture and immune system
- Prolonged life span-equivalent human 2.5X
- Similar HSPC phenotype/frequency



Aged Macaques Have Human-type ARCH Mutations

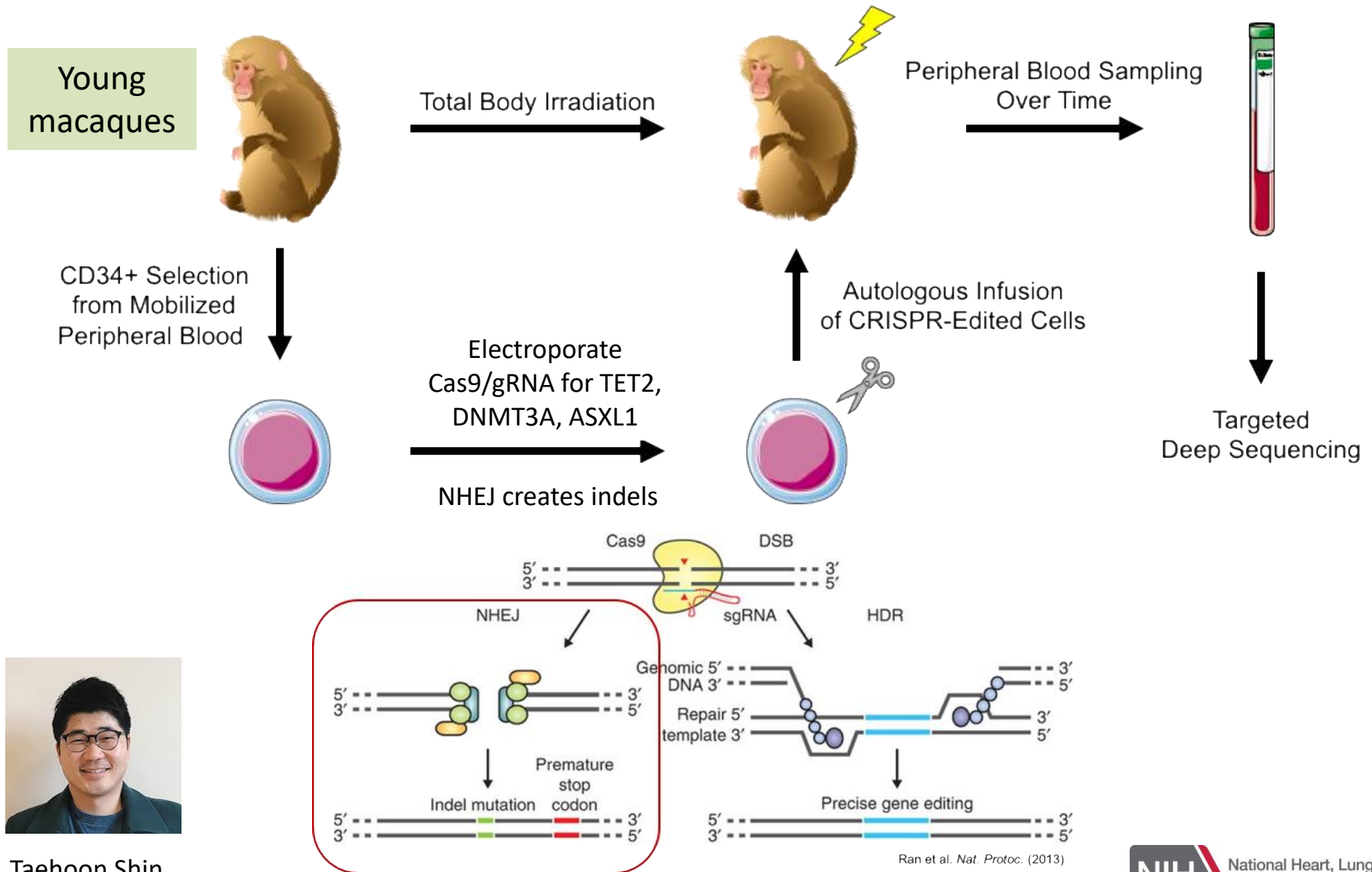
- N=53 aged macaques studied to date
- Panel of 56 genes previously associated with ARCH, AML, MDS and other blood cancers
- 27% have coding region somatic mutations, median age 28



Yifan Zhou

- Commonly-mutated genes mirror human ARCH
 - DNMT3A and TET2 most common
 - Expand over time
- Suggest similar HSPC dynamics result in ARCH in both species

Generation of a Engineered Macaque Model



Taehoon Shin

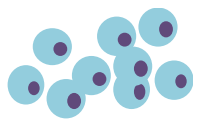
Gradual and Marked Expansion of TET2-mutated HSPC clones



ZL26

Cas9/gRNA
Ribonucleoprotein (RNP)

CD34+ HSPC



80%

20%

Multiplexed gRNAs
DNMT3A, TET2, ASXL1

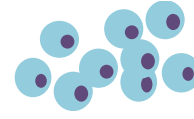
AAVS1 (control locus)



ZL39 / ZH63

Cas9/gRNA
Ribonucleoprotein (RNP)

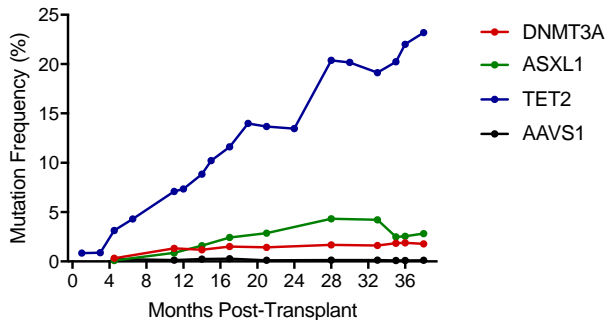
CD34+ HSPC



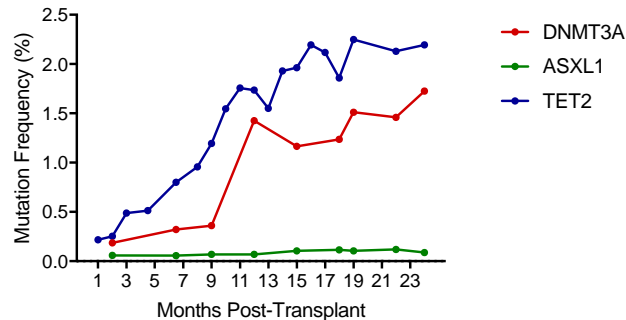
100%

Multiplexed gRNAs
DNMT3A, TET2, ASXL1

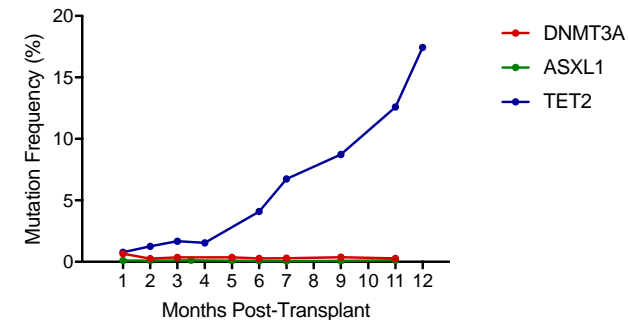
ZL26 Granulocytes



ZL39 Granulocytes

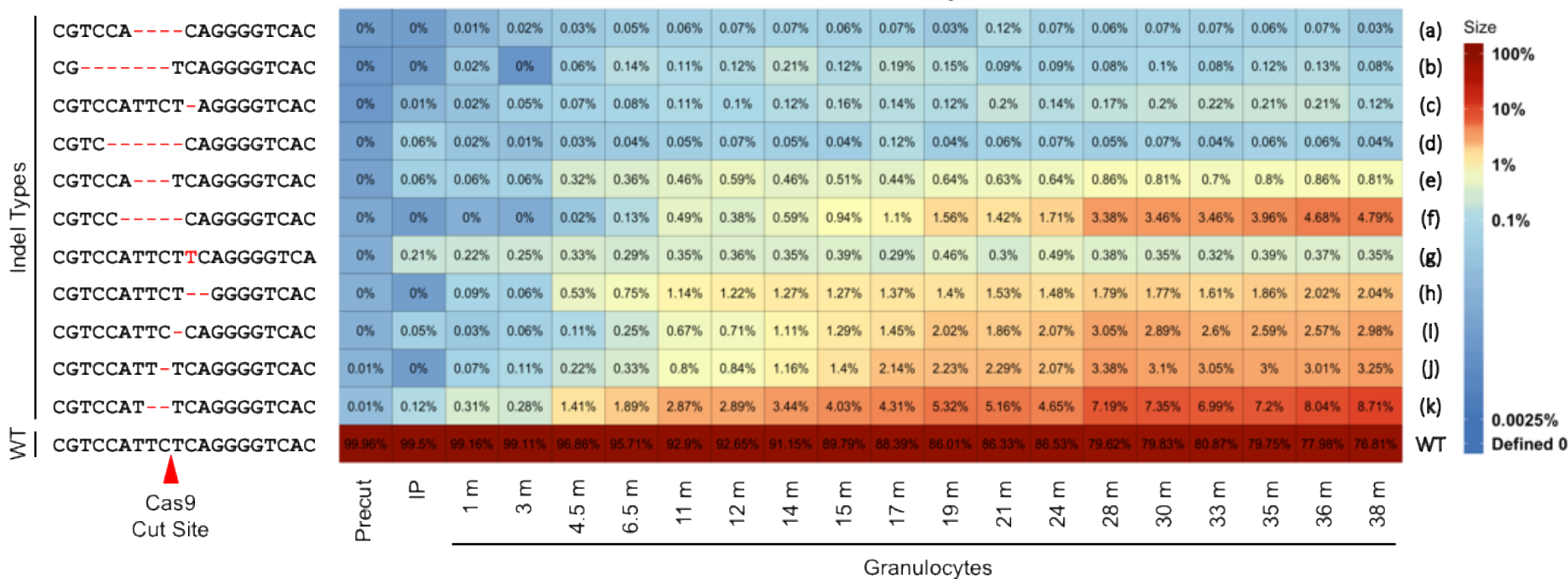


ZH63 Granulocytes



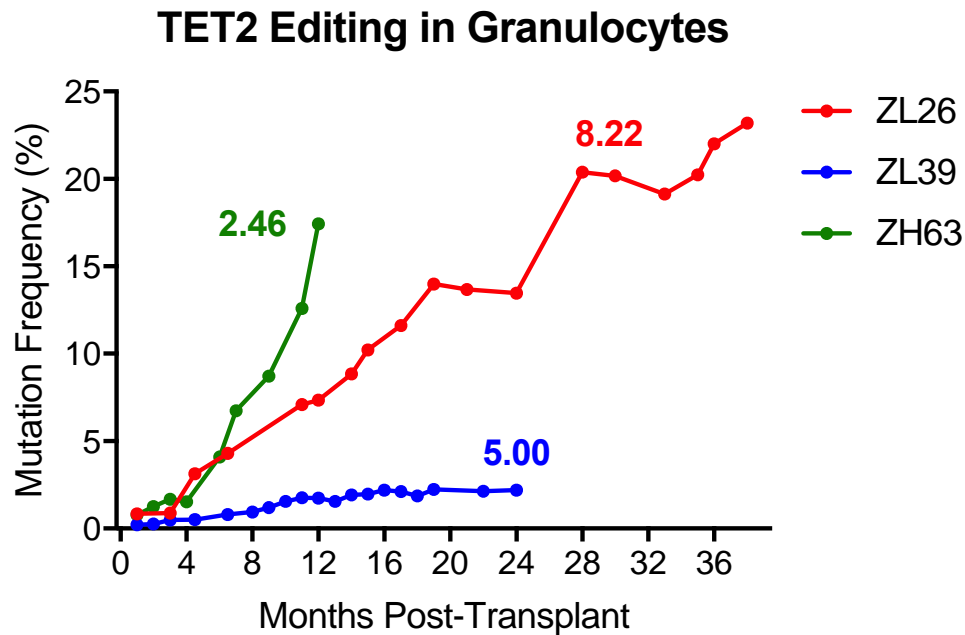
Gradual and Marked Expansion of TET2-mutated HSPC clones

ZL26 Granulocyte - TET2



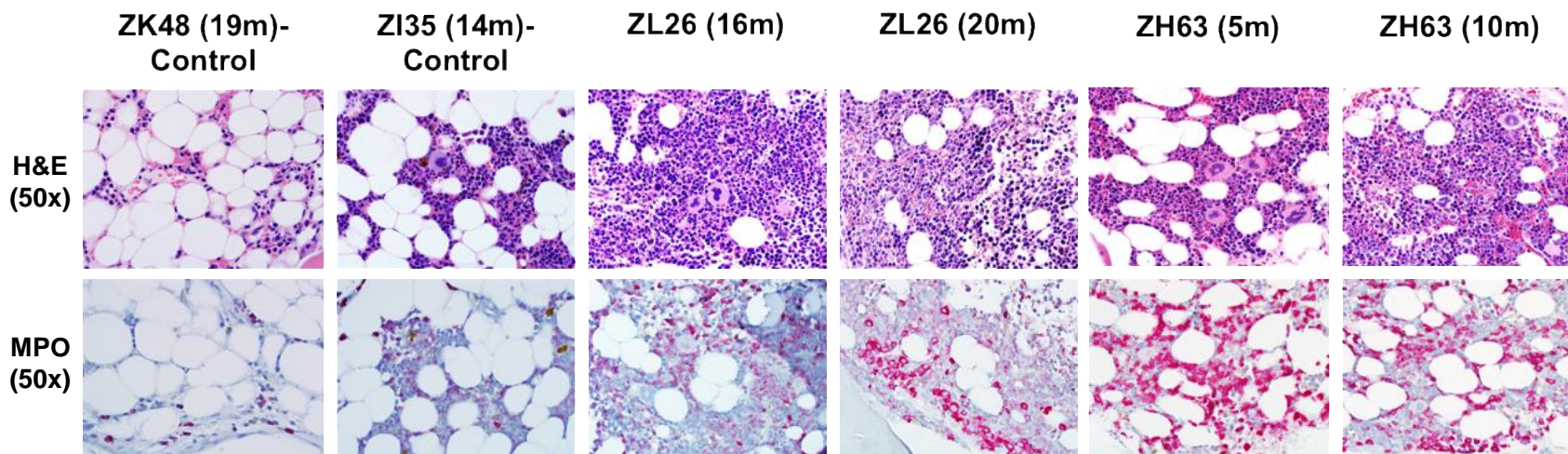
- Multiple expanding clones with predicted LOF (loss of function) mutations
- No "second hits" necessary

Gradual and Marked Expansion of TET2-mutated HSPC clones

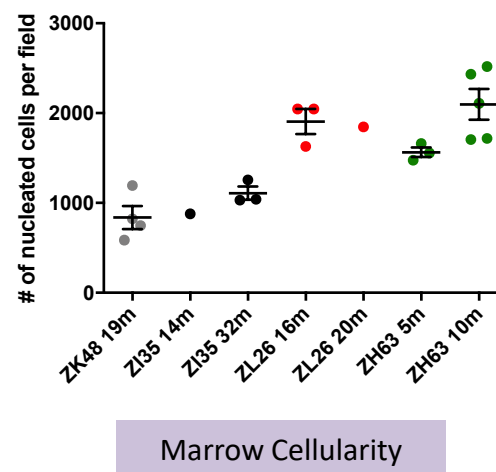


- TET2 mutated clonal expansion rates among the three macaques varied markedly
- Specific host intrinsic factors such as other genetic differences, age of the microenvironment, or presence of inflammation could impact on rate of TET2 mutant clonal expansion
- Variability of clonal expansion in humans beginning to be linked to inflammation, smoking, other environmental and intrinsic factors

Bone Marrow Hypercellularity in TET2-mutated Macaques



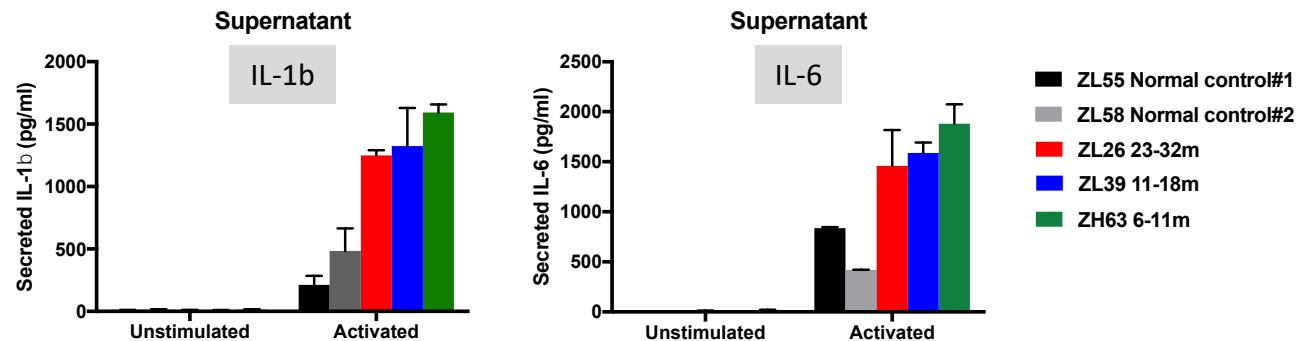
- Compared to controls same time from transplantation
 - Increased cellularity
 - Myeloid shift
 - No dysplasia or increased blasts
- Normal blood counts in all three macaques to date



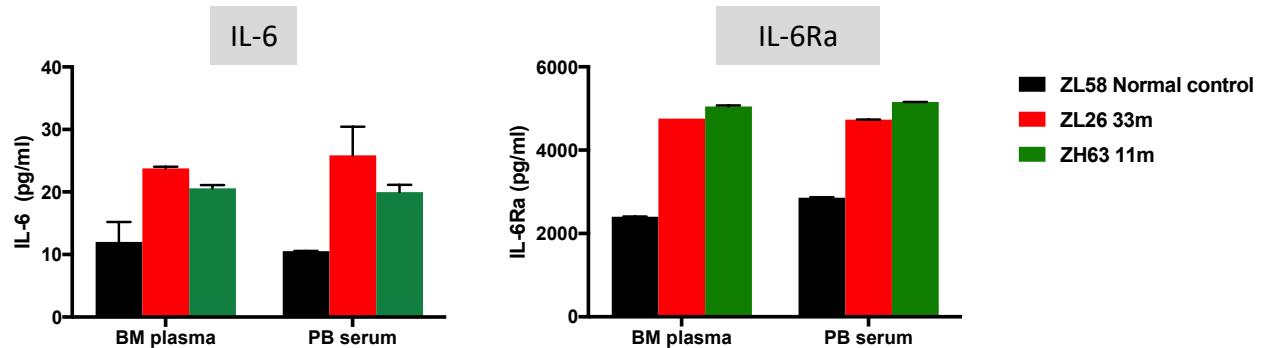
Increased Inflammatory Cytokine Expression and Secretion

- RNASeq on myeloid marrow progenitors from the edited macaques
 - Control vs TET2 mutant
 - Increased inflammatory cytokine and chemokine expression from TET2 mutant cells

Macrophages in culture

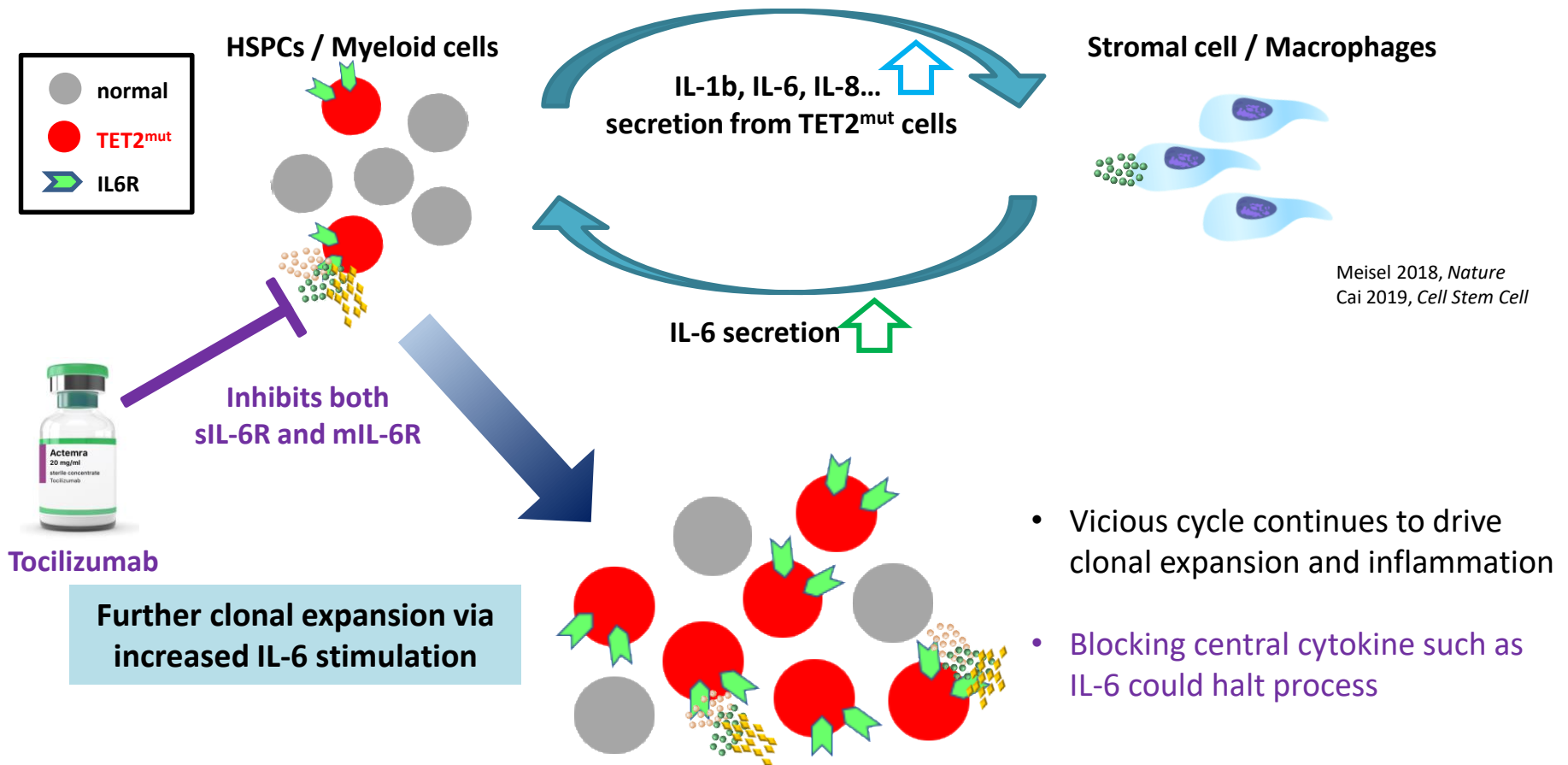


In Serum

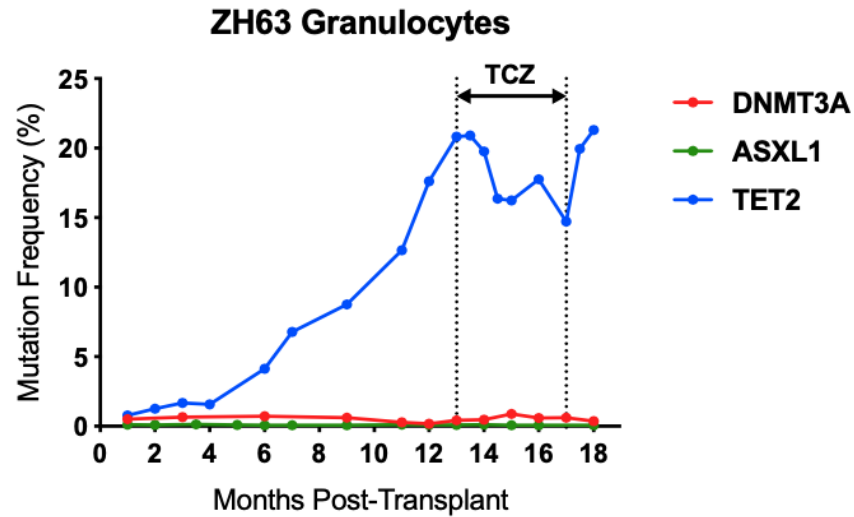


- Even in macaque ZL39 with low allele fraction

Model for Inflammation and Clonal Expansion



Impact of Tocilizumab on TET2 Mutation Frequency

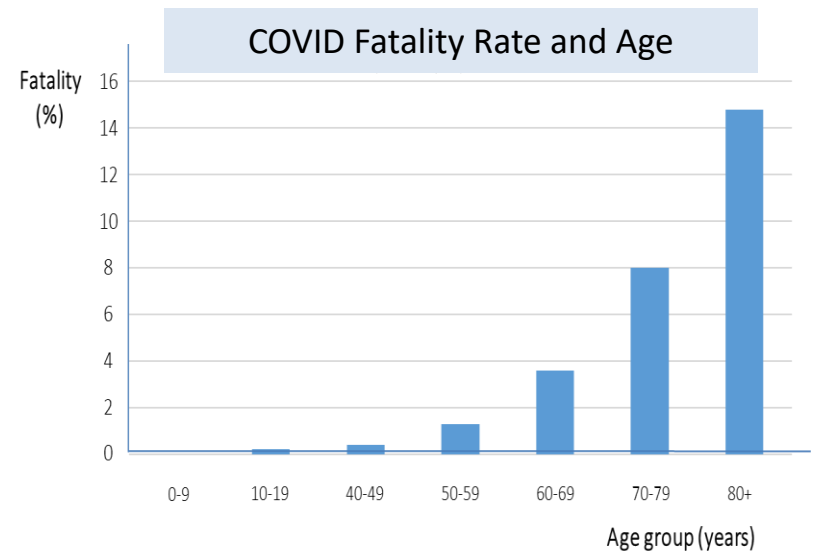
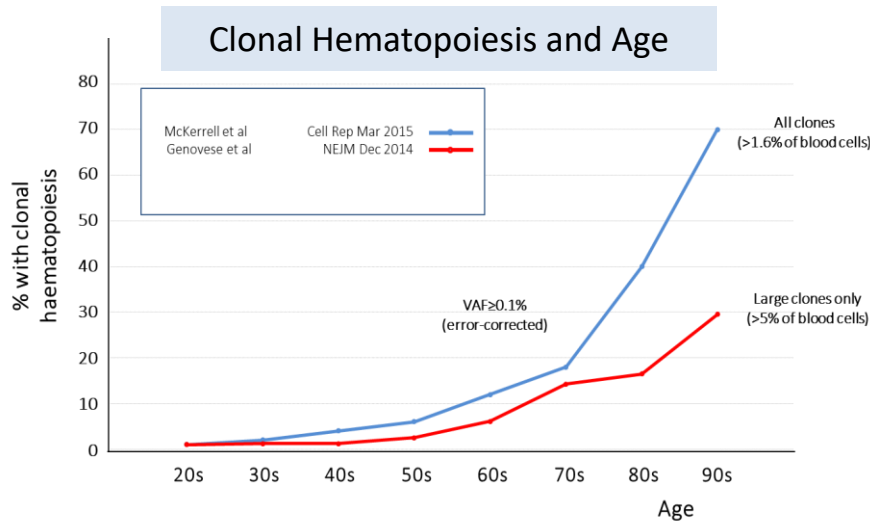


- Actual decrease in TET2 indels
- The frequency of DNMT3A and ASXL1 indels did not change
- Clonal expansion begins again with TCZ discontinuation

Summary Macaque ARCH Models

- Loss-of-function (LOF) in TET2 is sufficient to drive rapid and reproducible clonal expansion of HSPCs in rhesus macaques
- An aged microenvironment is not necessary for clonal expansion, however, variable rates of expansion between individual animals suggest role for extrinsic factors
- All ARCH/CHIP mutations are not alike-DNMT3A and ASXL1 to date associated with slower or no clonal expansion
 - DNMT3A mutant clones may required aged microenvironment
- This model allows preclinical testing of interventions to stop clonal expansion and downstream consequences

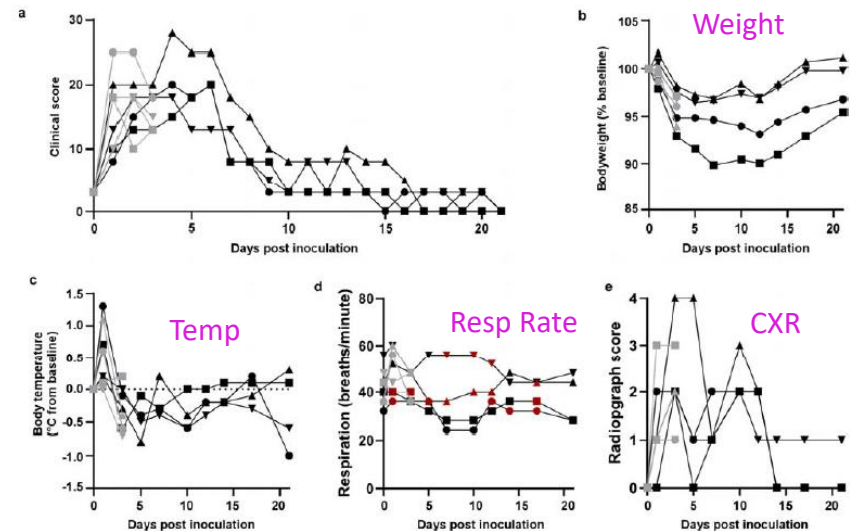
Age Relationships of ARCH and COVID



- COVID pts die from late hyperinflammatory tissue and organ damage
 - IL1b, IL6 implicated
- “Stochastic” highly heterogeneous progression to this outcome
 - “known” risk factors predict only 2/3rds of cases in some series
- *TET2*, *DNMT3A*, *JAK2* ARCH mutations all result in hyperinflammatory phenotype
- Could ARCH mutations predispose to these poor COVID outcomes?
 - Collaborative project with George Vassiliou to sequence patients with different severities of COVID for ARCH mutations

Relevant Animal Model for Late Hyperinflammatory COVID-19 Disease?

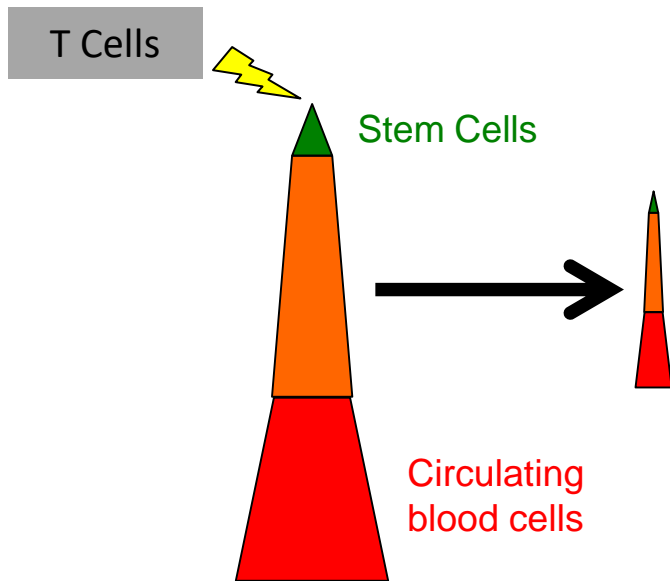
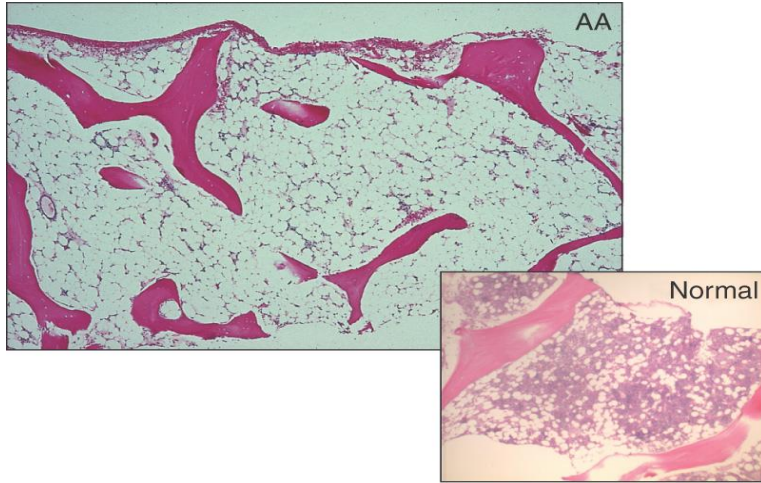
- Engineered infectable mice
- Rhesus macaques being utilized for therapy and vaccine development (Munster et al, bioXrV, 2020)
- Mild disease in young macaques
- No mortality, late hyperinflammation



Munster et al BioXrV preprint

- Use ARCH macaques as a model for hyperinflammatory COVID
 - If enhanced late hyperinflammatory disease, utilize to study pathophysiology and interventions

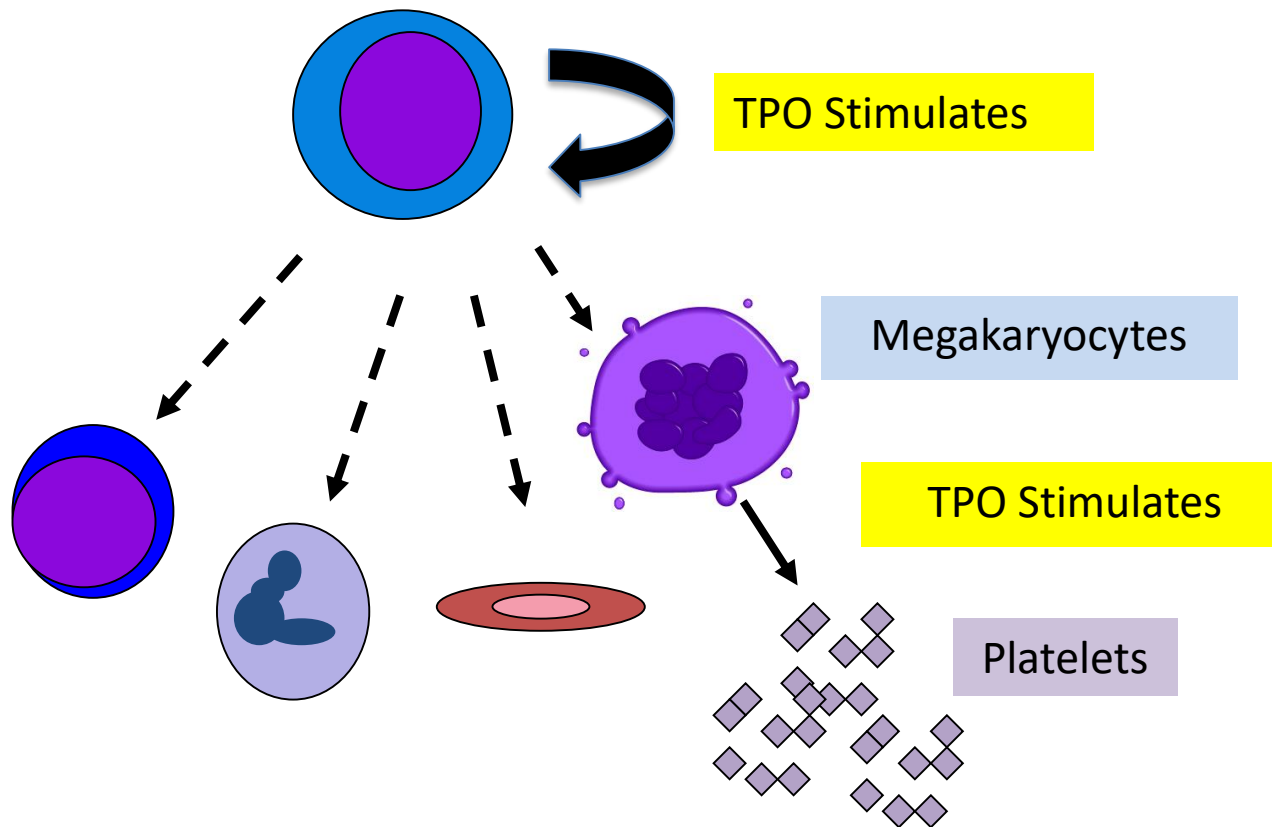
Aplastic Anemia: A Stem Cell Deficiency Disorder



Hematopoietic Compartment

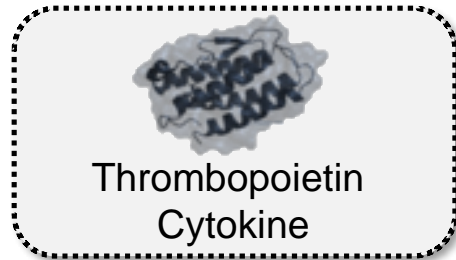
- Profound marrow hypocellularity
- Severe pancytopenia-anemia, bleeding, infections
- Acquired AA-**autoimmune** T cell attack on HSPC
- Treatment for acquired AA
 - Allogeneic HSC transplant
 - ATG/CSA immunosuppression response in 60%
 - Relapses up to 30%, clonal progression 20%
 - Non-responders or refractory relapsing patients had no effective therapeutic options beyond supportive care

Thrombopoietin (TPO) and HSPC

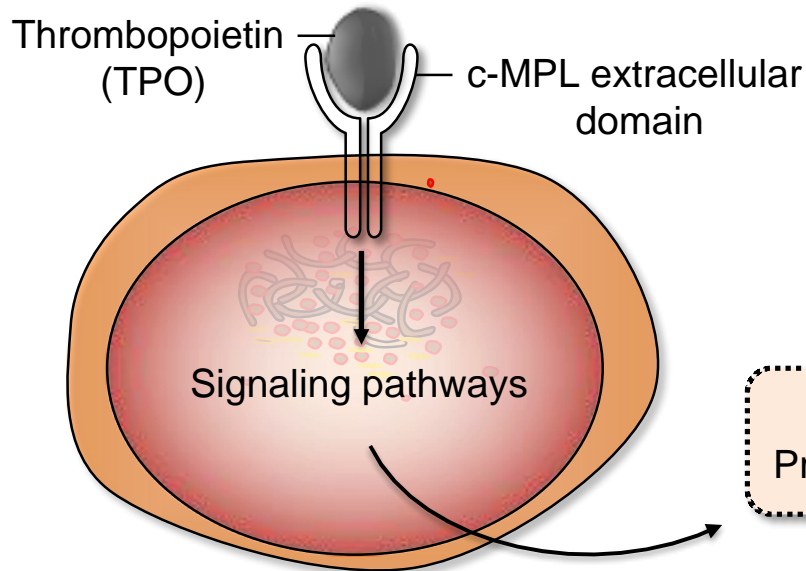
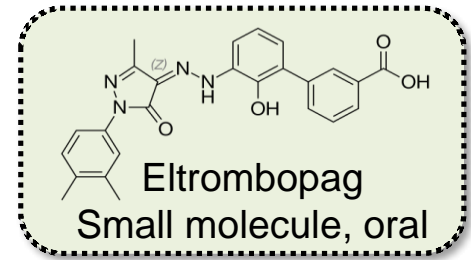


- Potent activity of TPO in cycling/transducing primitive HSPCs in vitro
 - *Wu et al Mol Ther 2000; Takatoku et al JCI 2001*
- HSC failure and pancytopenia in mice/humans with Tpo or Mpl loss of function mutations

Eltrombopag: Small Molecule TPO Agonist

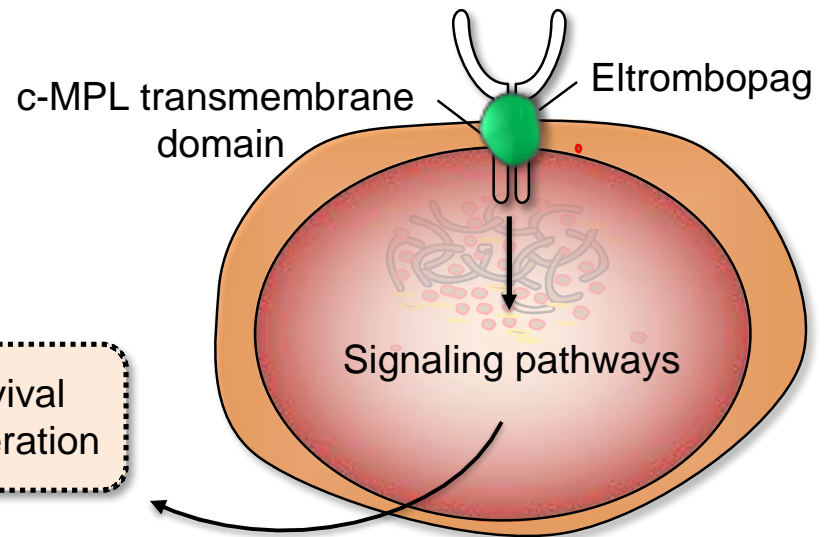


EPAG approved
for ITP 2008



Hematopoietic stem/progenitor cell
(HSPC)

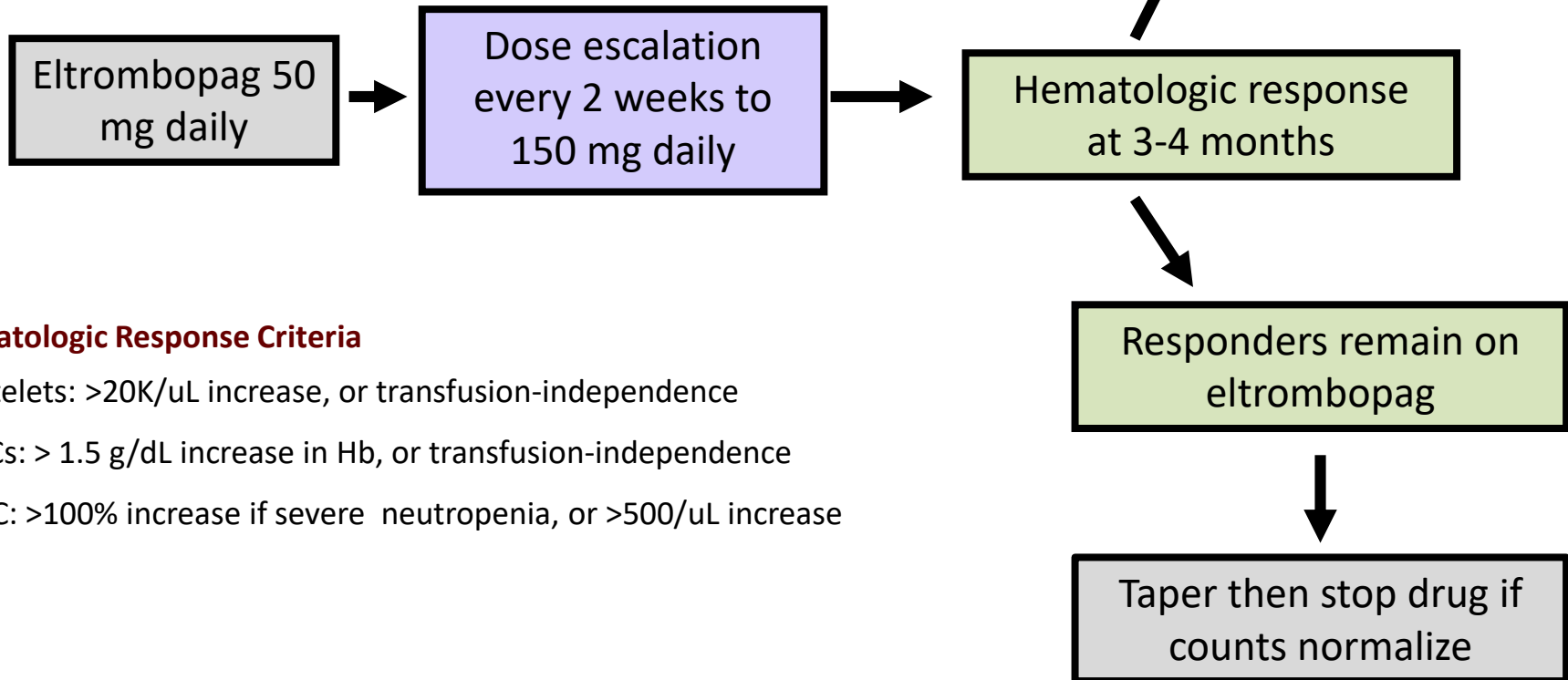
Survival
Proliferation



Hematopoietic stem/progenitor cell
(HSPC)

EPAG for Refractory SAA: Initial Trial

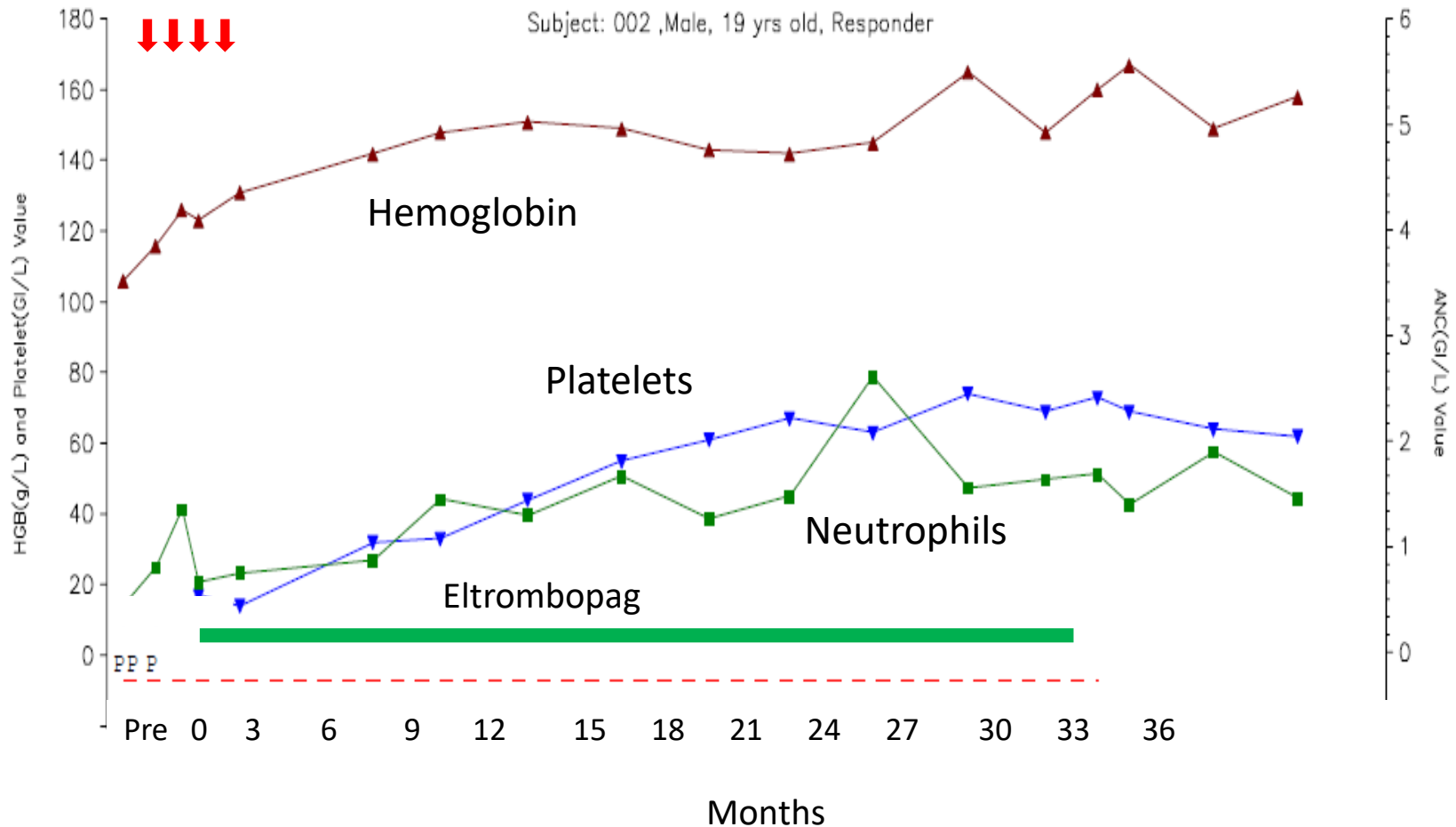
- 45 SAA patients
- Refractory to ATG/CSA



Hematologic Response Criteria

- Platelets: >20K/uL increase, or transfusion-independence
- RBCs: > 1.5 g/dL increase in Hb, or transfusion-independence
- ANC: >100% increase if severe neutropenia, or >500/uL increase

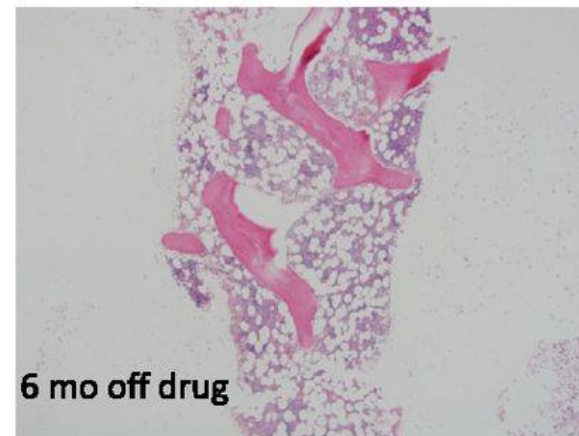
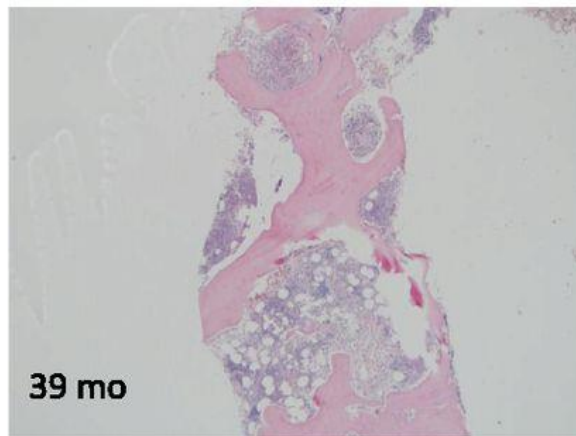
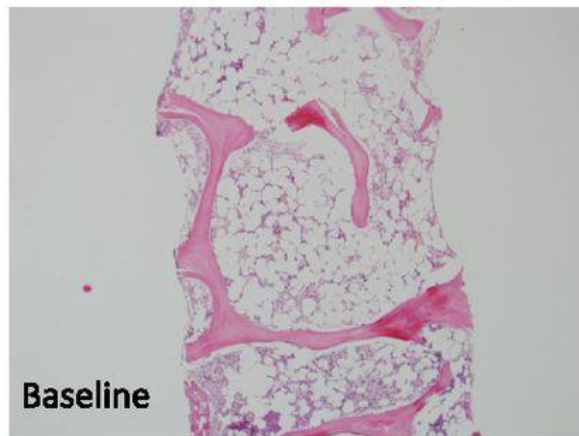
Effect of Eltrombopag on Blood Counts



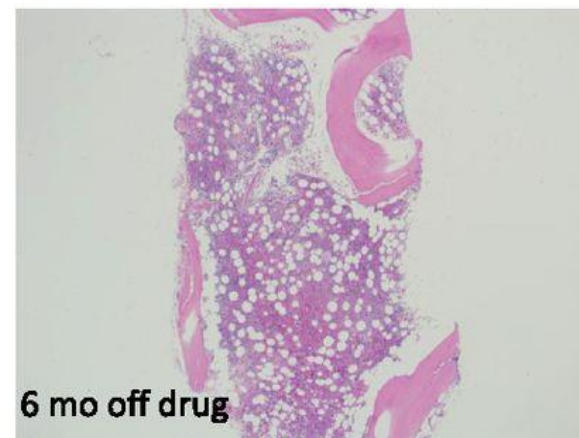
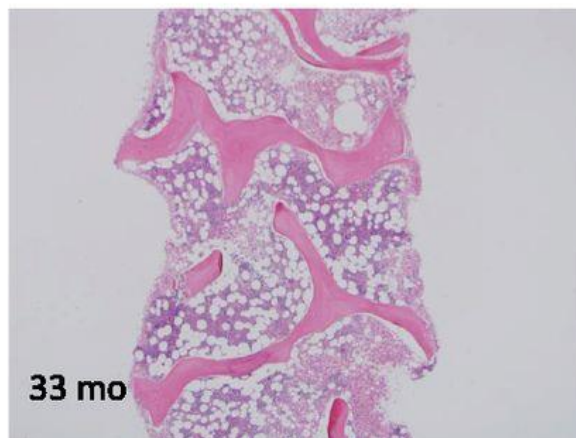
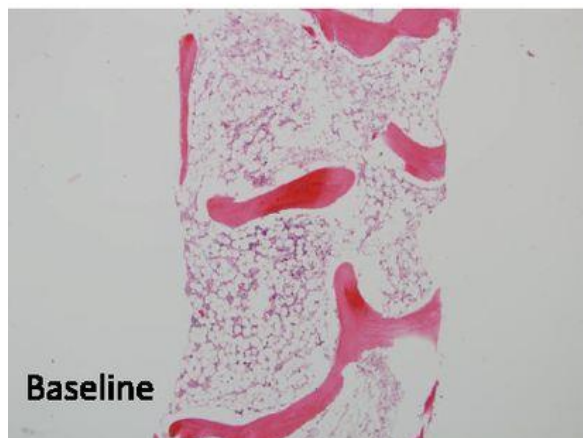
Hemoglobin----- Platelets----- Neutrophils----- Transfusions ↓↓↓↓

Normalization of Marrow

Patient 1



Patient 2



Eltrombopag and Improved Hematopoiesis in Refractory Aplastic Anemia

Matthew J. Olnes, M.D., Ph.D., Phillip Scheinberg, M.D., Katherine R. Calvo, M.D., Ronan Desmond, M.D., Yong Tang, M.D., Ph.D., Bogdan Dumitriu, M.D., Ankur R. Parikh, M.D., Susan Soto, B.S.N., Angelique Biancotto, Ph.D., Xingmin Feng, M.D., Ph.D., Jay Lozier, M.D., Ph.D., Colin O. Wu, Ph.D., Neal S. Young, M.D., and Cynthia E. Dunbar, M.D.

- 40-50% response rate
- Responses clinically-significant, **multi-lineage**, stable for up to 11 years
- Sustained off eltrombopag once robust count recovery
 - ¼ required EPAG reinstitution-all responded again
- Minimal toxicity: transient transaminitis, nausea, diarrhea, occasional severe skin rash

Regular Article

CLINICAL TRIALS AND OBSERVATIONS

BLOOD, 20 MARCH 2014 • VOLUME 123, NUMBER 12

CME Article



Eltrombopag restores trilineage hematopoiesis in refractory severe aplastic anemia that can be sustained on discontinuation of drug

Ronan Desmond,¹ Danielle M. Townsley,¹ Bogdan Dumitriu,¹ Matthew J. Olnes,² Phillip Scheinberg,³ Margaret Bevans,⁴ Ankur R. Parikh,¹ Kinneret Broder,¹ Katherine R. Calvo,⁵ Colin O. Wu,⁶ Neal S. Young,¹ and Cynthia E. Dunbar¹



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GSK/Novartis transaction

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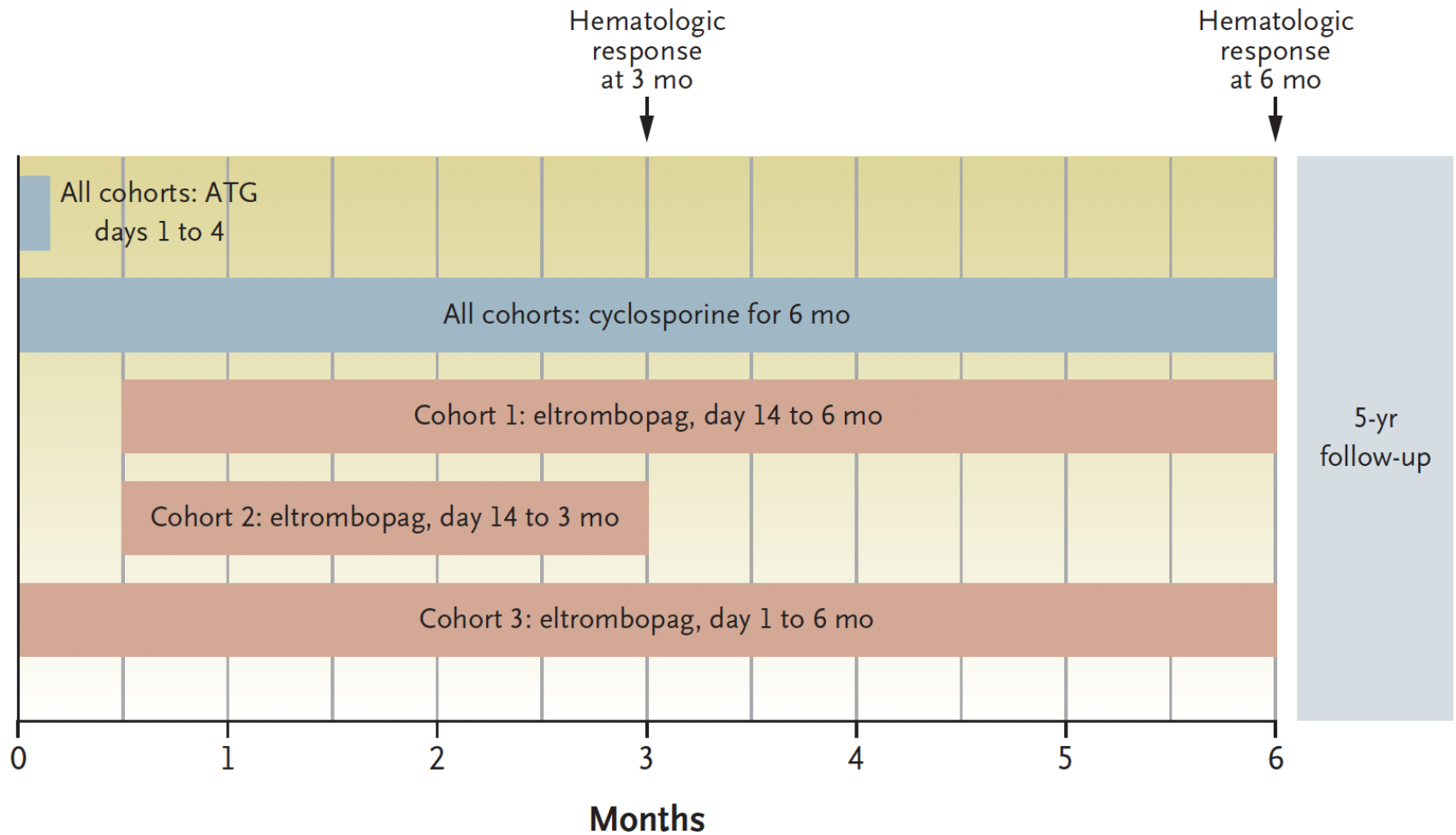
GSK receives FDA approval of an additional Promacta® (eltrombopag) indication for use in patients with severe aplastic anaemia (SAA) who have had an insufficient response to immunosuppressive therapy (IST)

26 August 2014

Issued: London, UK

- FDA approval 2014
- First new drug for AA in 30 years

EPAG Added to Standard ATG/CSA for Treatment-Naïve Severe Aplastic Anemia



Hematologic Response and Complete Response Rates Improved

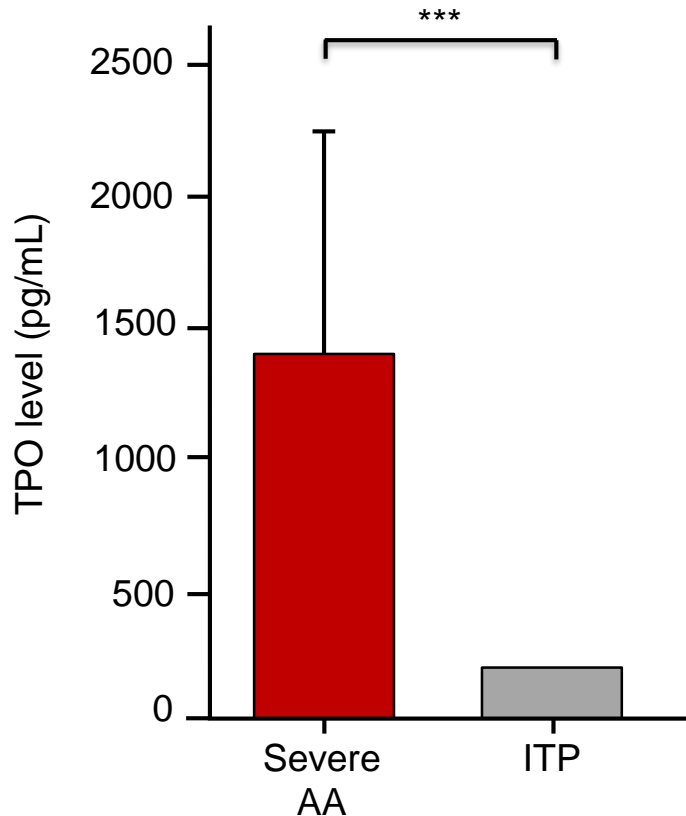
| | Cohort 1 N=30 | Cohort 2 N=31 | Cohort 3 N=31 | All Cohorts N=92 | Historic rates N=388* |
|----------|------------------|------------------|------------------|---------------------|--------------------------|
| | N (%) | N (%) | N (%) | N (%) | |
| 3 months | | | | | |
| OR | 23 (77) | 24 (77) | 27 (87) | 74 (80) | 60% |
| CR | 5 (17) | 8 (26) | 15 (48) | 28 (30) | 8% |
| 6 months | | | | | |
| OR | 24 (80) | 27 (87) | 29 (94) | 80 (87) | 63% |
| CR | 10 (33) | 8 (26) | 18 (58) | 36 (39) | 12% |

Townsley DM, et al. NEJM 2017; 376:1540-50.

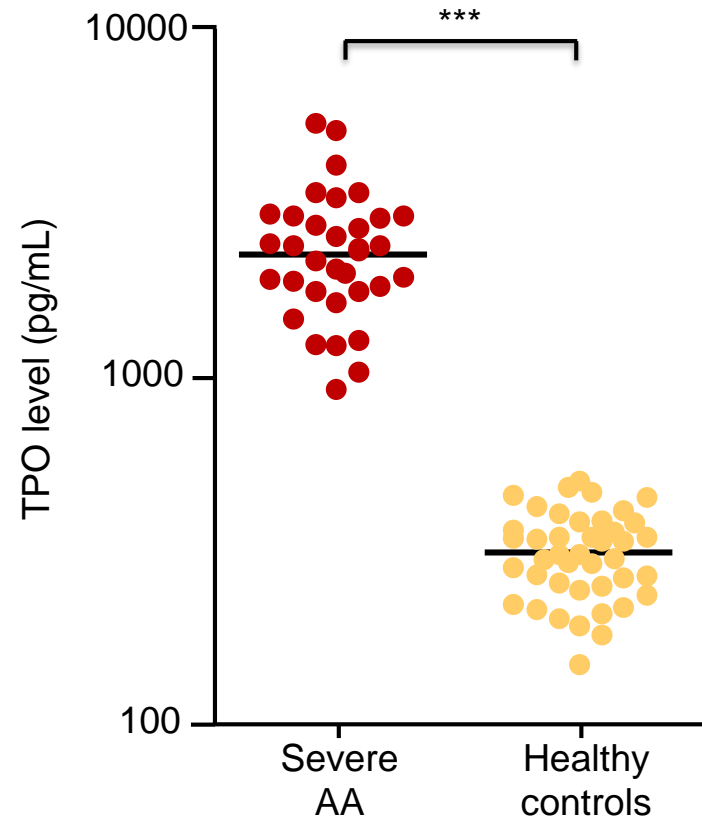
- FDA approval 2018 for new onset severe aplastic anemia

Serum TPO Levels in Thrombocytopenia

Endogenous TPO levels are already markedly elevated in patients with severe aplastic anemia (AA)



Emmons R et al., Blood 87:4068 (1996)



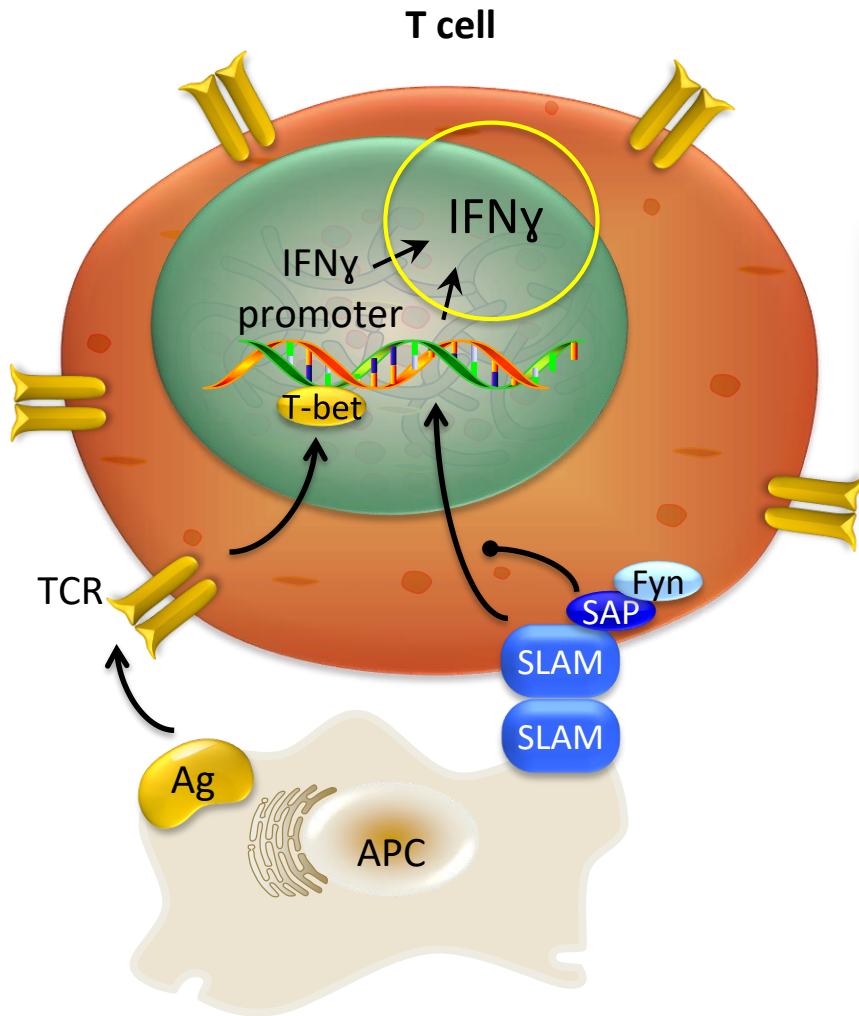
Feng X et al., Haematologica 96:602 (2011)

How Does EPAG Improve Hematopoiesis Despite Elevated TPO Levels in Marrow Failure?



Dr. Andre Larochelle
Tenure-Track Investigator NHLBI

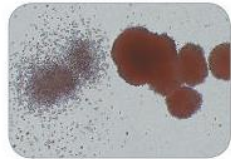
Inflammatory Cytokines are Elevated in SAA



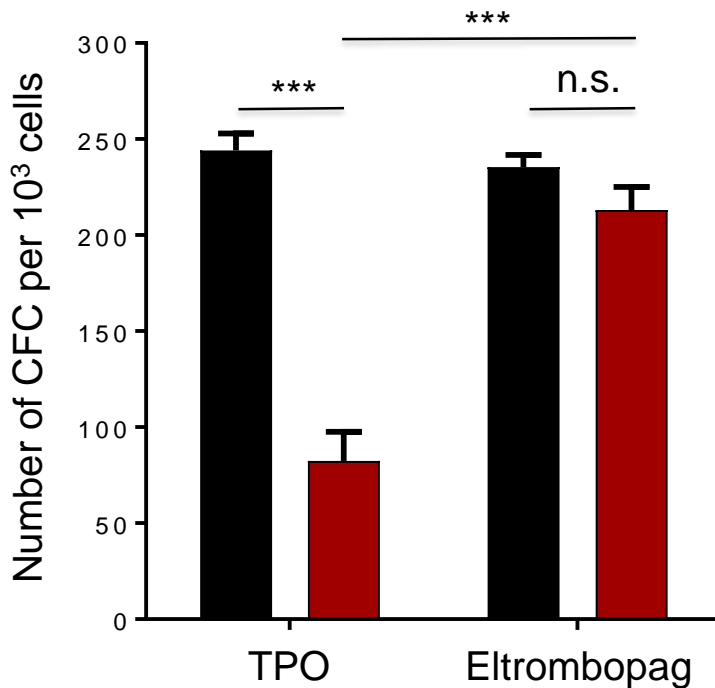
| | Interferon (IU/mL) Bone marrow | Interferon (IU/mL) Peripheral blood |
|------------------------------|-----------------------------------|--|
| Aplastic patients (n=8) | 203 ± 54 | 53 ± 32 |
| Normal individuals (n=16) | 41 ± 54 | <10 |
| P value | 0.001 | 0.001 |

EPAG but not TPO Maintains HSPC in the Presence of IFN γ

Colony forming cell (CFC) assay



■ Without IFN γ
■ With IFN γ

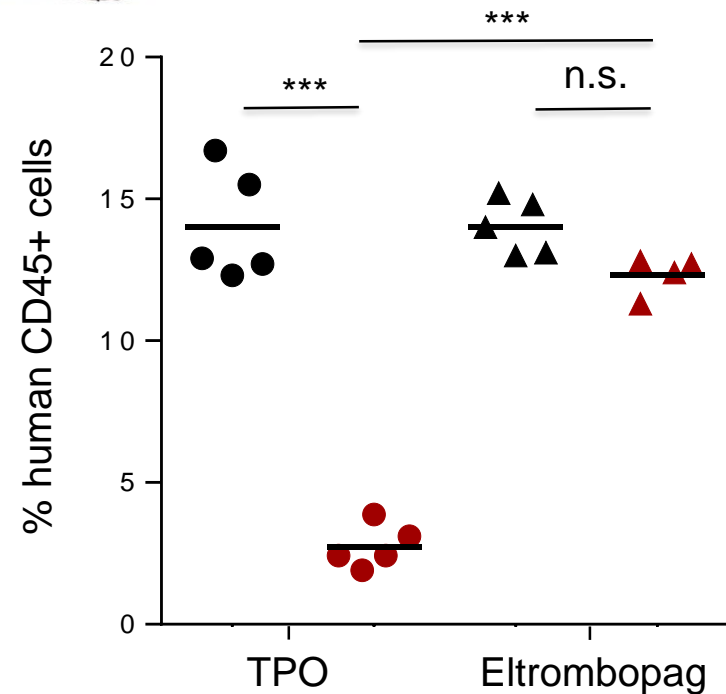


n=3

Transplantation into NSG mice



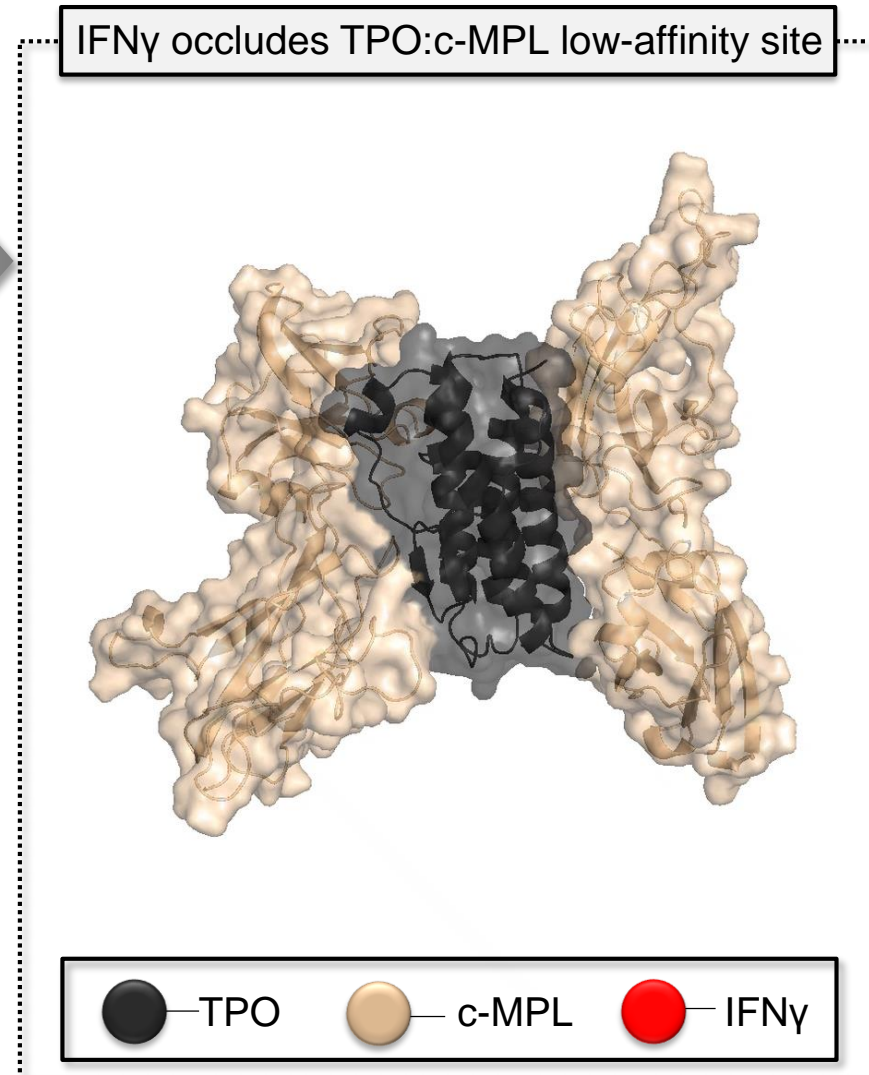
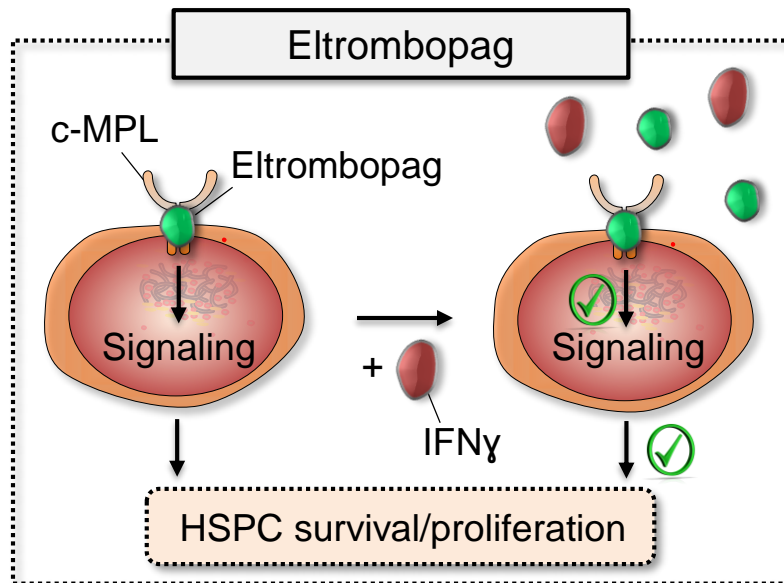
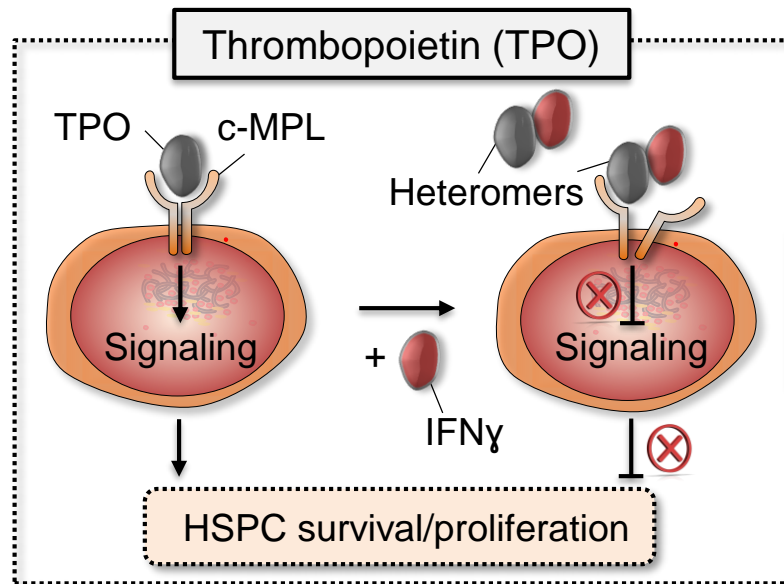
■ Without IFN γ
■ With IFN γ



CD45+ = human cell engraftment (HSCs)

Model of IFN γ -Mediated Bone Marrow Failure

Signaling Inhibition by TPO:IFN γ Heteromers in Human HSPCs





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